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It is characteristic of Science and Progress that they continually open new fields to our vision.—Pasteur.

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PROGRESS REPORT FOR 1920 COMMITTEE ON BACTERIOLOGICAL TECHNIC

H. J. CONN, *Chairman*, K. N. ATKINS, I. J. KLIGLER, J. F. NORTON, AND G. E. HARMON

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Committees dealing with various matters of bacteriological technic have been appointed in the past by this Society and by other organizations interested in bacteriology. There have been, for example, the committees on standard methods of water analysis and on standard methods of milk analysis of the American Public Health Association, also our committee on methods of milk analysis to coöperate with the latter, and our committee on the descriptive chart. With the exception of the committee on the descriptive chart, all these committees have had for their chief function the standardization of technic and the establishment of official methods. Even the committee on the descriptive chart at first entertained the plan of establishing official methods for pure culture study; but as the work of the committee progressed, it proved that it might have a wider usefulness as an agency through which different procedures might be compared and their relative merits for different purposes established without giving official standing to any one technic. So important did this particular function of the committee appear, and so many similar problems along other lines were called to its attention, that finally the committee on the chart resigned and a new committee was appointed in December 1919 to take up in the same manner various points of technic of interest to bacteriologists. A continuation of the work on the chart was assigned to this committee as part of its function.

The logic of such a committee as a part of our Society is evident. The other bodies with committees on bacteriological technic are in general interested in official control work and

desire methods that give uniform and reasonably reliable results with as little labor as possible, rather than methods giving the most accurate scientific data. As a society of bacteriologists, however, we should be interested in the accuracy of technic rather than in simple and inexpensive methods.

WORK ON THE DESCRIPTIVE CHART

The use of the descriptive chart has lately come to be mainly for instruction purposes. Hence the recent committee on the chart drew up a folder especially designed for instruction. There has been considerable demand for this chart, but two modifications have been quite generally called for: its condensation into smaller space, and the omission of the old and illogical group number. To see how generally this opinion is held among bacteriologists, an enquiry was addressed to each instructor who has ordered the Society charts during the last two years. The replies received have almost unanimously been in favor of a single sheet chart without the group number. These two modifications, it was pointed out, would make the chart more useful not only to instructors but to investigators as well. Accordingly both modifications have been adopted in the new chart which the committee is proposing to the Society this year, together with various minor changes which it is hoped will be found to be improvements.

The new chart is like the instruction folder in the omission of the detail which made the old card poorly adapted to the instruction laboratory, but a few of the more commonly used tests, omitted from the instruction chart, such as that for indol, are included on the new form. By the use of finer type and the reduction of the space left for sketches, all this material has been condensed on two sides of an $8\frac{1}{2}$ by 11 inch sheet. Nevertheless, some blank space is still retained for sketches and for recording the results of special tests. The group number, as such, is omitted entirely; but all the useful purposes of the group number are retained by adopting a new form of marginal characterization. In place of the group number, an "Index number" has been

substituted, the object of which is merely to assist the student in filing a large number of the completed charts according to the salient characteristics of the organisms described on them. Its use, however, is optional; it is plainly stated to be intended for index purposes only; and as it does not contain the generic symbol, there is no danger of its suggesting to the novice that it is intended to supplant the specific name of an organism.

If this chart meets the approval of the Society, it will be printed and will be ready for distribution at about the time that this report appears in the Journal. The old charts will still be kept on sale as long as there is any demand for them. All the charts may be obtained from the chairman of this committee (address Geneva, N. Y.). A sample copy of the new chart will be sent to anyone on request.

METHODS OF PURE CULTURE STUDY

The committee on the descriptive chart prepared two or three reports on methods of pure culture study (1918, 1919, 1920), which the present committee plans to keep up to date. To do this, new methods are being investigated that they may be published in future reports. The methods at present under investigation are: methods of determining acid production from sugars and other carbon compounds; methods of determining diastatic action on starch; modifications of the Gram stain. A preliminary publication on the first of these problems has already been made this year by Conn and Hucker (1920). There is nothing yet ready for publication on the other problems, further than the material which appeared in the 1919 report of the committee on the descriptive chart.

METHODS OF COUNTING BACTERIA

There is no phase of bacteriological technic that has been given more attention by scientific organizations than methods of counting bacteria. The reason for this is the importance from the public health standpoint of knowing the number of bacteria in any food or drink for human consumption. It must not be

forgotten, however, that there is one other equally important object in counting bacteria, for it is only by this means that we can determine the abundance of the organisms in any particular habitat—a problem of value from the standpoint of pure science. This latter aspect of the matter especially concerns this Society rather than organizations interested in disease or public health. The very fact that the subject is being so thoroughly investigated from the standpoint of the sanitarian makes it all the more important that it be studied by this Society also. Standardization of methods—which has been the chief aim of other organizations taking up the matter—tends to prevent progress by fixing the technic. To counteract this tendency, the committee on technic plans to compare the various methods of counting bacteria, laying chief stress upon their accuracy, rather than upon their adaptability to routine use.

There are three criteria by which methods of counting bacteria can be judged: (1) agreement of duplicate determinations; (2) size of the counts obtained; and (3) actual accuracy of the counts. The first of these, agreement of duplicate determinations, is the object desired in control work, where incomplete counts are entirely suitable, provided a uniform fraction of the total number of bacteria is counted. Size of the counts is the most commonly used criterion as to the relative merits of different methods of counting, because it is generally recognized that ordinary counts are but partial ones and the presumption is that the higher count is the more nearly correct. Actual accuracy, however, although a far better criterion, is the hardest of all to apply, because counts may be too high instead of too low, and there is no absolute standard of comparison with which to check up results. Even the best bacterial counts are but estimates because the total numbers are too high to count with absolute accuracy, and high magnification is necessary to see the individuals; so the only way the accuracy of any one method may be determined is by comparing it with other methods and discounting the probable sources of error in each method.

There are three general types of methods by which bacteria may be counted: the dilution method, the plate method, and the

microscopic method. The dilution method (whereby a medium is inoculated with progressively decreasing quantities of the material under investigation until a dilution is reached too great to contain any bacteria) is cumbersome and is not applicable to many types of bacterial flora. The plate method and the microscopic method, each with various modifications, are in common use and can be applied to a great variety of bacterial habitats. Each method has its advantages and each its disadvantages; but by using both methods and properly comparing the results, it is possible to obtain very good information as to the actual number of bacteria in the material under investigation. An admirable investigation of this sort, of the methods of counting bacteria in milk, has recently been made by Breed and Stocking (1920).

Although milk has been investigated in this way more than any other material, there are other natural habitats of bacteria where it may be fully as important to know the best methods of determining the actual numbers of organisms present as well as to have official methods for routine use. There are, for example: water, soil, sewage, vaccines, and various foodstuffs, such as cheese, ketchup, butter, ice-cream, hamburg steak, dried egg powder, and so forth. The field is too broad to cover at once; but by attacking one problem at a time and by the eventual establishment of various sub-committees, it is hoped to round up the matter in time.

Shortly after the appointment of the committee, the problem of microörganisms in ketchup was referred to it. In this case the work is practically limited to the microscopic method, as the processing of the material kills the greater part of the organisms originally present. Counting is difficult, and yet results are important because they have already been used in the control of this food industry. It was pointed out to the committee that the industry would be glad to finance an investigation, but wanted it entirely free from their influence. (The name of the person or firm offering the money is not known to us.) The matter was turned over to the New York Agricultural Experiment Station, and it was found that they would gladly furnish the

facilities for the work, but did not wish to accept money from a commercial source. The Experiment Station, therefore, took it up with the National Research Council. Upon receiving the endorsement of the Research Council of our Society, the National Research Council agreed to act as an intermediary and to become responsible for the supervision of the investigation. In this way a responsible, scientific, and disinterested supervision of the work has been secured.

Although this work is no longer in the hands of the committee on bacteriological technic, and when completed will be published as an independent piece of investigation, it is given its place in this report because the problem was originally submitted to the committee and the investigation was planned as a part of the general committee program. It is regarded as merely a beginning. Other similar problems are to be investigated in the future. One that has already been referred to the committee is the counting of bacteria in vaccines and other similar preparations. Anyone interested in this matter is hereby urged to correspond with the chairman of this committee on the subject.

STANDARDIZATION OF STAINS

As this committee is primarily interested in the accuracy of technic, one of the first points that has been called to its attention is the inaccuracy of certain procedures (e.g., Gram stain) due to the present unreliability of dyes used in staining. It was suggested that the committee might undertake to test the various stains on the market and to certify the reliable products, also that it might do what it could to stimulate the production in America of dyes needed but not at present manufactured in this country. A circular letter was addressed to the members of the Society and there was found to be much interest, a considerable number of members volunteering to help in the work. The matter has also been discussed with certain producers and distributors of biological stains.

There is plainly a demand for work of this sort, and the committee is willing to undertake it if it can be properly organized.

Certain difficulties are in the way, in establishing satisfactory relations with commercial firms, and in securing the time and labor necessary to organize the work; but it is felt that these difficulties can be overcome. Further announcements will be made if the present plans develop.

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A STUDY OF THE VARIATIONS IN HYDROGEN-ION CONCENTRATION OF BROTH MEDIA

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At the present time it would seem scarcely necessary to lay emphasis upon the importance to bacterial growth and metabolism of the reaction of the environmental culture medium. That different degrees of acidity and alkalinity in media may profoundly influence the morphology, rate of fermentation, pigment production, growth, or viability of bacteria has been so thoroughly recognized that in the routine preparation of culture media as carried on in every bacteriological laboratory, the proper adjustment of reaction is carefully regulated. The use of scales of reaction such as that of Fuller, based upon adjustment to a definite "degree" of titratable acidity, has permitted a certain amount of uniformity, and in general, it may be said that these old titrimetric procedures have served a very useful purpose. But with the development, during the last few years, of the newer physicochemical conception of hydrogen-ion concentration the theory of titration has undergone a fundamental change. As a consequence many of the data obtained in earlier investigations are of little value, having been based upon unsound premises.

An adequate conception of the far-reaching biological effects of hydrogen-ion concentration may best be gained through a study of the classic works of Michaelis (1914),[1] Sörensen (1912, 1909a, 1909b) and Clark and Lubs (1917a, 1917b, 1917c). The following statement from the works of the last-named investigators will serve to emphasize the importance to the science of bacteriology of this modern conception of acidity and alkalinity:

[1] Bibliography is found at the end of the third article, in this series, p. 231.

Hydrogen-ion concentration influences the condition in solution of every substance with acidic or basic properties—native proteins and their hydrolytic products, amines and amides, carboxyl, sulphonic, and phenolic compounds, even alcoholic compounds, as well as many inorganic compounds. It has a large effect on the effective solubilities and dispersion of colloids, upon determining tautomeric equilibria, and in one way or another in governing the activity of catalysts such as hydrolytic enzymes and oxidases. One or the other of these effects, induced directly or perhaps indirectly by the hydrogen-ion concentration must impress bacterial life.

That the expression of reaction in terms of titratable acid or alkali does not adequately define the true reaction of a solution has perhaps best been brought out by W. M. Clark (1915a) in his admirable paper, "The 'reaction' of bacteriologic culture media." The objections to the older procedure may be summarized in a quotation from Clark and Lubs (1917a):

> The titrimetric method, designed originally for the quantitative estimation of strong acids and bases, cannot be applied to complex mixtures of very weak acidic and basic groups such as are found in the constituents of most culture media. In so far as the method is used to determine the "free acid" or to adjust to a certain degree of "free acid" it is an absolute failure when applied to culture media. There is however, an even more fundamental reason why the titrimetric method is inappropriate. Two media adjusted to the same degree of acidity may have widely divergent hydrogen-ion concentrations as shown by Clark (1915a).

With the development of the hydrogen electrode, making possible a direct measurement of hydrogen-ion concentration, some of the experimental and mathematical difficulties involved in the older methods were obviated, but there still remained to be elaborated some simpler and more rapid procedure that would be adapted to the adjustment of culture media and to the study of reaction changes in bacterial cultures. Guided by the earlier work of Friedenthal (1904), Salm (1904), Friedenthal and Salm (1907) who were the first to give a well worked-out series of indicators, Sörensen (1909a) in 1909 published his colorimetric method for determining hydrogen-ion concentrations. Since

this time a number of modified procedures have been suggested by Levy, Rowntree, and Marriott (1915), Hurwitz, Meyer and Ostenberg (1915, 1916); McLendon (1916); Barnett and Chapman (1918); Clark and Lubs (1916a, 1917a, 1917b, 1917c); Haas (1919); so that at the present time it is a relatively simple matter to prepare and have on hand in the ordinary bacteriological laboratory a suitable set of colorimetric standards for the measurement of the hydrogen-ion concentration of media and cultures. It is to Clark and Lubs (1917b, 1916b), Lubs and Clark (1915, 1916) that we are especially indebted for several new and valuable indicator substances as well as for a careful study of the ranges and usefulness of an entire set of indicators for the examination of biological fluids.

Deeleman (1897) in 1897, using the titration procedure, noted that media underwent certain changes in reaction during sterilization and sought to avoid such variation through the addition of proper amounts of sterile acid or alkali to the autoclaved material. Hesse (1904) used the same procedure in the adjustment of his media and further emphasized the fact that only that type of glassware which yields no alkali should be employed for containers, to prevent the increase in alkalinity that otherwise might occur. According to Sörensen (1909a) however, such factors as alkalinity from glassware and CO_2 from the atmosphere exert only slight effects if the medium in question is properly buffered. Using the titration method, Anthony and Ekroth (1916) attempted to bring media to a stable reaction by repeatedly alkalinizing and autoclaving, but were unable to produce such a stabilized condition even after many additions of alkali, supplemented by a total of fourteen hours autoclaving. They explain the change as due to the formation of acid principles through hydrolysis. In one case five times the quantity of base needed was added through an error, with the result that after several sterilizations the reaction of the broth fell to the required level. Wright (1917) has suggested that the amount of alkali indicated by titration is never sufficient to bring about a complete neutralization of the medium, it being always necessary to add a considerable excess over the amount indicated. On the other hand, Noyes

(1916) states that properly prepared media do not increase appreciably in acidity when the length of sterilization is increased or when repeated autoclavings are carried out. It is a known fact that many proteins may exist in solution only between certain limits of hydrogen-ion concentration and that slight changes, at or near the critical zones, cause the formation of precipitates. This phenomenon occurs in peptone solutions and as Kligler (1917) has shown it is possible to establish the limits of P_H which determine precipitation for each brand of peptone.[2] Cook and Lefevre (1918) showed that as much as 12 per cent of peptone may be lost through precipitation depending on whether this material were added previous to coagulation and filtration or subsequently. That a change in P_H accompanies such a precipitation in media has been found by Clark (1915a) who reported a fall in P_H of 0.80 (from 8.52 to 7.72) in an infusion broth containing 0.5 per cent K_2HPO_4. Itano (1916a) using the hydrogen electrode in his P_H measurements, was able to establish a rough correlation between the changes in P_H of an extract broth upon autoclaving and the increase in COOH groups as determined by the formol titration of Sörensen. Strangely enough the changes in P_H reported by Itano were always of the nature of an increase in alkalinity, and with this there appeared an increase in formol-titrating nitrogen, indicating that hydrolysis had occurred. As a result of boiling the broth for forty-five minutes this observer found that the material became stable as regards further changes in P_H. This last experiment, however, was tried only on media adjusted between P_H 5.45 and 6.88. By sterilizing the constituents of his media separately it was possible to adjust to the desired P_H and obtain values which remained fairly constant throughout the entire experiment. Norton (1919) has reported that appreciable changes in the reaction of neutral and alkaline media, but little variation in the acid range, result from sterilization. Davis (1920), in recognition of the possibility of a change in the P_H of media adjusted in the alkaline range, has suggested that for the proper preparation of a glucose broth of P_H 8.0–8.2 reaction it is well to

[2] The symbol P_H of Sörensen is used throughout to designate the hydrogen-ion concentration.

bring the material to an initial P_H of 8.6. Davis also emphasizes the superiority of the autoclave over the Arnold for media sterilization pointing out that prolonged heating is always to be avoided in order that the vitamine or hormone content may not undergo destruction. On the other hand, Fennel and Fisher (1919) report that in the preparation of over one hundred lots of beef infusion broth the initial P_H of 7.8 did not show variation as a result of autoclaving. In connection with his study of the effect of initial reaction of a medium upon *Corynebacterium diphtheriae*, Bunker (1919) noted certain reaction changes in his media upon sterilization. The variations appeared almost entirely on the alkaline side and were always noted as increases in acidity.[3] Very recently, Grace and Highberger (1920b) have carried out experiments with extract broth which seem to indicate that changes in reaction upon sterilization may not be of any greater order than are the changes which a medium may undergo simply upon standing, following autoclaving. The variations of greatest magnitude occurred in the alkaline range and all changes were toward a more acid reaction. No consistent tendencies could be detected, therefore it was not possible to come to definite conclusions as to the reasons for the observed changes. However, the possibilities of the influence of glass and atmospheric CO_2, as well as of slow hydrolysis, were suggested.

Early in the present investigation it was noted that culture media (broth) adjusted to definite P_H levels underwent changes in reaction upon autoclaving, thus rendering difficult the preparation of broth of desired reaction. Consequently it was considered important to investigate these changes with a hope of finding an explanation and perhaps of discovering some means of avoiding them.

METHODS AND TECHNIC

Standard solutions

All solutions were prepared according to the methods outlined by Clark and Lubs (1916a, 1917a) from boric acid and salts which

[3] The term acidity in the present paper signifies true acidity as expressed in terms of P_H.

had been recrystallized three to five times. Triple distilled water served as solvent. The stock solutions, as well as the standard buffer mixtures, were kept in heavily paraffined, glass-stoppered bottles. Check determinations on the mixtures at the outset and after a period of seven months showed that the standard buffers, from bottles in which the paraffin was not broken, had remained constant in P_H in spite of the fact that molds had developed in some of the liquids. Sörensen (1909a) reported a similar observation on solutions after nine months standing. The desired P_H ranges and the solutions used in their preparation are given below:

Solutions	P_H
M/5 Potassium acid phthalate, M/5 NaOH	4.0–5.8
M/5 KH_2PO_4, M/5 NaOH	5.8–7.6
M/5 H_3BO_3, M/5 KCl, M/5 NaOH	7.8–9.0

Indicators

The indicator solutions were the following:

CHEMICAL NAME	COMMON NAME	CONCENTRATION IN 50 PER CENT C_2H_5OH	RANGE P_H
		per cent	
Ortho carboxy benzene azo di-methyl aniline	Methyl red	0.02	4.4–6.0
Di brom ortho cresol sulphon phthalein	Brom cresol purple	0.04	5.2–6.8
Phenol sulphon phthalein	Phenol red	0.02	6.8–8.4
Thymol sulphon phthalein (alkaline range)	Thymol blue	0.04	8.0–9.6

Color standards

Color standards were prepared by adding 0.3 cc. of the required indicator solution to 5 cc. of the buffer mixture. Tubes of colorless glass and uniform bore, 4 by $\frac{3}{8}$ inches were used for the color standards as well as for the test liquids. Fresh standards were made up each week, as fading is apt to occur if the solutions are allowed to stand for a longer period. This is most pronounced in the methyl red series and least noticeable in the brom cresol purple series.

Colorimetric determination of hydrogen-ion concentration

In properly buffered solutions it is possible partially to eliminate such factors as color and turbidity by diluting the test fluid with water. Preliminary tests showed that with broth and cultures it was possible to dilute 1cc. of the material with 4 cc. of distilled water without altering the hydrogen-ion concentration. Accordingly this technic was employed in all the determinations. Freshly boiled and cooled distilled water was used for diluting as preliminary tests had shown that unboiled water gave slightly lower P_H readings. The P_H of the water itself was usually found to rise from 4.8 to 6.8 upon boiling, probably due to liberation of carbon dioxide. To eliminate factors of color and turbidity more completely Walpole's (1911) method of superposition was used by employing the comparator block described by Dernby and Avery (1918). All determinations were carried out at room temperature. The limit of error in the readings was 0.1 P_H.

The adjustment of broth media

One cubic centimeter of the broth was diluted with distilled water (freshly boiled and cooled). Two acid solutions and two basic solutions were kept on hand. They were N/1 HCl and an exact 1:10 dilution of the same; N/1 NaOH and an exact 1:10 dilution. A specially made micro burette, of 1 cc. capacity and graduated to 0.01, contained the diluted acid or base. This was added to the tube containing the medium, water, and 0.3 cc. of the proper indicator solution until the color produced therein exactly matched that of the color standard of desired P_H. The reading on the micro burette was then taken and by calculation the amount of stock acid or base needed to adjust the total amount of broth was determined. Following the addition of the acid or base to the entire lot of medium a check determination was always carried out. The broth was autoclaved at 15 pounds for twenty minutes. In case this caused the formation of a precipitate the medium was filtered and subjected to a second autoclaving for twenty to thirty minutes at 10 pounds pressure. The low pressure prevents a second precipitation of the medium. The

P_H should always be taken on the broth following the final autoclaving as well as at the outset of any given experiment. The reason for this will appear in the experiments about to be described.

In case the broth was to contain a sterile sugar this was added aseptically in 10 per cent solution to the sterile medium to avoid any possibility of splitting the sugar through heating. This procedure is especially important if the broth is adjusted in the acid or alkaline range as it is a known fact that glucose and other sugars are altered by heating with even small amounts of acid or base (Mathews, 1916). Furthermore, Mudge (1917) has observed an increased titratable acidity when sugars, at least disaccharides, are autoclaved with media. By adding the sugar aseptically in concentrated solution no change in reaction was ever noted.

Experiment I. The extent of the changes in hydrogen-ion concentration which broth media adjusted to different initial P_H levels undergo upon autoclaving and standing

The unadjusted broth was divided into portions of 75 cc. which were brought to values ranging from P_H 5.0 to 9.0 at intervals of 0.4. Five cubic centimeter amounts were then tubed and autoclaved at 15 pounds for fifteen minutes after which they were allowed to cool and P_H readings taken. The tubes comprising each lot were divided into three sets, one of which was allowed to remain at room temperature, another was placed in the ice chest, while the third was incubated at 37°. After standing at these temperatures for intervals of two, seven, and fourteen days tubes were removed and P_H determinations made.

Five series were carried through and the data obtained are to be found in tables 1 to 5.

SERIES	COMPOSITION OF BROTH	RESULTS IN TABLE
I	Beef infusion	1
II	Beef extract	2
III	Bacto beef	3
IV	Beef infusion (repetition of I)	4
V	Beef extract (repetition of II)	5

Reference to tables 1 and 4, containing data for the two beef infusion series, reveals differences in P_H changes as a result of autoclaving. Whereas every tube of series I showed an increased acidity upon sterilization, the tubes of series IV from 5.0 to 5.8 inclusive exhibited a decrease in acidity; those of P_H 6.1–7.3 suffered no alteration in reaction, while those lying in the 7.8–8.9 range showed a definite increase in acidity. Upon standing, the greatest changes in both series are manifest in the 8.6 and 9.0

TABLE 1

Experiment I. Changes in reaction upon autoclaving and standing. (Beef infusion broth)

Composition:

Distilled water	1000 cc.
Chopped lean beef	300 grams
Peptone (Parke, Davis & Co.)	10 grams
NaCl	5 grams

BEFORE AUTOCLAVING	AFTER AUTOCLAVING	ROOM TEMPERATURE AFTER DAYS			ICE CHEST AFTER DAYS			INCUBATOR AFTER DAYS		
		2	7	14	2	7	14	2	7	14
5.0	4.8	4.8	4.4	4.7*	4.8	4.4	4.7*	4.8	4.4	4.7*
5.3	5.0	4.9	4.8	5.0*	4.9	4.8	5.0*	5.0	4.8	5.0*
5.8	5.6	5.5	5.4	5.3	5.5	5.4	5.3	5.5	5.4	5.3
6.2	5.9	5.8	6.0	5.8	5.8	6.0	5.9	5.8	6.0	5.9
6.5	6.2	6.2	6.4	6.2	6.1	6.3	6.2	6.2	6.4	6.2
7.1	6.9	6.8	7.0	7.0	6.8	6.8	7.0	6.7	6.9	7.0
7.3	7.1	7.1	7.2	7.3	7.1	7.2	7.3	7.1	7.3	
7.8	7.3	7.3	7.6	7.6	7.3	7.5	7.6	7.4	7.6	7.6
8.1	7.8	7.7	7.9	7.8	7.7	7.8	7.8	7.7	7.9	7.8
8.6	8.4	8.4	8.4	8.1	8.4	8.4	8.1	8.4	8.4	8.1
9.0	8.6	8.6	8.5	8.2	8.5	8.5	8.2	8.6	8.6	8.3

* The unexpected increase in alkalinity may have been more apparent than real due to a fading of the standard buffer mixtures of the methyl red series.

tubes. These changes are in the nature of increases in acidity and are as great in magnitude as those produced by autoclaving. A possibility of this sort has apparently been overlooked by many observers. No differences worthy of mention appear as a result of storing the broth under different conditions of temperature.

Passing to the two beef extract series (tables 2 and 5) a remarkably small number of alterations are notable in one case (V). An increase in acidity of 0.2 P_H occurred in the two lots of highest

P_H, namely the 8.6 and 9.0 tubes. These two lots were practically the only ones to exhibit changes upon standing, the 9.0 registering an acidity change of 0.7 P_H after fourteen days standing. In series II (table 2) decreases in acidity are noted in the acid and alkaline ranges upon autoclaving while within the range 6.6–7.3 the broth remained unchanged. In every lot of this series the acidity increased upon standing, the greatest changes occurring in the

TABLE 2

Experiment I. Changes in reaction upon autoclaving and standing. (Beef extract broth)

Composition:

Distilled water	1000 cc.
Liebig's beef extract	3 grams
Peptone (Parke, Davis & Co.)	10 grams
NaCl	5 grams

BEFORE AUTOCLAVING	AFTER AUTOCLAVING	ROOM TEMPERATURE AFTER DAYS			ICE CHEST AFTER DAYS			INCUBATOR AFTER DAYS		
		2	7	14	2	7	14	2	7	14
5.0*	5.2*	5.3	4.6	4.7	5.2	5.1	4.8	5.3	4.8	4.8
5.3*	5.4*	5.6	4.7	4.8	5.5	5.1	4.8	5.5	4.8	4.8
5.8	6.0	6.1	5.2	5.2	6.1	5.2	5.2	6.1	5.2	5.2
6.2	6.4	6.3	5.6	5.6	6.3	5.4	5.6	6.3	5.6	5.6
6.6	6.6	6.6	6.2	6.3	6.6	6.3	6.4	6.6	6.5	
7.0	7.0	7.0	6.8	6.8	7.0	6.8	6.8	7.0		
7.3	7.3	7.3	7.0	7.0	7.3	7.0	7.0	7.3	7.0	7.0
7.7	7.9	7.9	7.5	7.8	7.9	7.4	7.6	7.9	7.4	7.8
8.0	8.3	8.4	7.8	7.9	8.4	7.7	7.9	8.4	7.6	7.9
8.7*	8.9*	8.5	8.4	8.6†	8.6	8.4	8.6†	8.6	8.6	8.7†
9.0*	9.2*	8.8	8.6	8.8†	9.0	8.4	8.6†	9.0	8.6	9.0†

* Precipitate.

† A slight fading of the standard buffer mixtures of the thymol blue series may have occurred thus accounting for the apparent increase in alkalinity.

more acid and alkaline ranges. Here, as previously mentioned in the case of beef infusion broth, the changes on standing seem to be independent of the environmental temperature.

The results in the bacto-beef series (table 3) are similar to those noted in the case of beef infusion. A decreased acidity in general appears in the range, 5.0–6.2, the 6.6–8.2 tubes remain practically unchanged, while the most alkaline members, 8.6 and 9.0 show increases in acidity upon autoclaving. Upon standing at

the three different temperatures the same general tendencies as have been observed in series IV may be noted.

It appears that there is no marked consistency in the variations which a given type of broth medium may exhibit as a result of autoclaving and standing. The same conclusions have been reached by Grace and Highberger (1920b) working with beef extract broth. Itano (1916a) however, reported only decreases in acidity in lots of extract broth adjusted throughout a wide range

TABLE 3

Experiment I. Changes in reaction upon autoclaving and standing. (Bacto-beef broth)

Composition:

Distilled water	1000 cc.
Bacto-beef	50 grams
Peptone (Parke, Davis & Co.)	10 grams
NaCl	5 grams

BEFORE AUTOCLAVING	AFTER AUTOCLAVING	ROOM TEMPERATURE AFTER DAYS			ICE CHEST AFTER DAYS			INCUBATOR AFTER DAYS		
		2	7	14	2	7	14	2	7	14
5.0*	5.4*	5.5	5.4	5.6	5.5	5.4	5.7	5.4	5.5	5.7
5.4*	5.2*	5.7	5.8	5.6	5.7	5.7	5.5	5.8	5.7	5.7
5.8*	6.3*	6.1	6.0	5.9	6.3	6.0	5.9	6.1	5.9	5.8
6.2	6.6	6.6	6.3	6.2	6.6	6.5	6.4	6.4	6.3	6.2
6.6	6.6	6.6	6.5	6.4	6.6	6.5	6.4	6.6	6.5	6.5
6.9	7.0	7.0	6.9	6.8	7.0	6.9	6.8	7.0	7.0	6.8
7.3	7.5	7.4	7.4	7.3	7.5	7.1	7.5	7.4	7.4	7.5
7.8	7.8	8.0	7.9	7.8	7.9	7.8	7.7	7.9	7.9	7.8
8.2	8.2*	8.2	8.1	7.9	8.2	8.0	7.9	8.2	8.1	7.9
8.6	8.4*	8.3	8.3	8.2	8.4	8.2	8.2	8.4	8.4	8.3
8.9*	8.6*	8.5	8.2	8.3	8.5	8.2	8.2	8.6	8.3	8.6

* Precipitate.

of P_H. His medium contained 2 per cent peptone which, as is well known, acts as a strong buffer. By sterilizing the components separately he was able to avoid anything more than slight alterations in reaction. No data were collected relative to the possibility of changes upon standing. The discrepancies appearing in the present beef infusion series were not so unexpected when it is considered that two different lots of beef were employed in their preparation, but the lack of uniformity in the changes

registered by the two beef extract series is not explainable upon such a basis for the same components were used in the preparation of each.

The remainder of the work has consisted of attempts to determine the causative factors in these reaction changes in order that some procedure might be devised to obviate the effects produced.

Although certain investigators have pointed out that the glassware employed may exert an effect upon the reaction of the con-

TABLE 4

Experiment I. Changes in reaction upon autoclaving and standing. (Beef infusion broth)

Composition:

Distilled water	1000 cc.
Chopped lean beef	300 grams
Peptone (Parke, Davis & Co.)	10 grams
NaCl	5 grams

BEFORE AUTOCLAVING	AFTER AUTOCLAVING	ROOM TEMPERATURE AFTER DAYS			ICE CHEST AFTER DAYS			INCUBATOR AFTER DAYS		
		2	7	14	2	7	14	2	7	14
5.0*	5.3*	5.2	5.3	5.3	5.2	5.3	5.3	5.2	5.3	5.3
5.4*	5.6*	5.5	5.6	5.5	5.5	5.6	5.4	5.4	5.5	5.4
5.6	5.8	5.6	5.8	5.8	5.6	5.8	5.8	5.6	5.8	5.8
5.8	5.9	5.9	6.0	6.0	5.9	6.0	6.0	5.9	6.0	6.0
6.1	6.1	6.1	6.2	6.2	6.1	6.2	6.2	6.1	6.2	6.2
6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5
7.0	7.0	6.9	7.1	7.1	6.9	7.0	7.1	6.9	7.0	7.1
7.3	7.3*	7.2	7.4	7.3	7.2	7.3	7.3	7.2	7.3	7.3
7.8	7.6*	7.5	7.6	7.6	7.4	7.5	7.6	7.4	7.5	7.6
8.0	7.9*	7.8	7.9	7.9	7.7	7.9	7.9	7.8	7.9	7.9
8.6*	8.5*	8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.3
8.9*	8.6*	8.4	8.4	8.3	8.4	8.4	8.3	8.4	8.4	8.3

* Precipitate.

tained media, experience in this laboratory has not borne out these contentions. In the course of the present work it has almost invariably been found that P_H determinations on a given medium distributed in different tubes check closely. Consequently this factor has at no time been seriously considered as even partially contributory to the reaction changes encountered.

It has been emphasized that in the very large majority of cases the reaction change was in the direction of an acidity increase

and further that the degree of variation upon standing was usually as great as upon autoclaving. In view of those findings the possibility of an absorption of sufficient CO_2 from the atmosphere to account for the changes noted was considered. Experiments II and III were carried out to decide this point.

TABLE 5

Experiment I. Changes in reaction upon autoclaving and standing. (Beef extract broth)

Composition:

Distilled water	1000 cc.
Liebig's beef extract	3 grams
Peptone (Parke, Davis & Co.)	10 grams
NaCl	5 grams

BEFORE AUTOCLAVING	AFTER AUTOCLAVING	ROOM TEMPERATURE AFTER DAYS			ICE CHEST AFTER DAYS			INCUBATOR AFTER DAYS		
		2	7	14	2	7	14	2	7	14
4.8*	4.8*	4.8	4.8	5.0	4.8	4.8	5.0	4.8	4.8	4.9
5.0*	5.0*	5.1	5.2	5.1	5.1	5.2	5.1	5.1	5.2	5.2
5.6	5.6	5.6	5.6	5.5	5.6	5.6	5.5	5.6	5.5	5.5
5.8	5.9	5.9	5.8	5.8	5.9	5.8	5.8			
6.1	6.2	6.1	6.0	6.4	6.1	6.0	6.0			
6.5	6.5	6.5	6.3	6.4	6.5	6.3	6.4	6.5		
6.9	7.0	7.0	7.0	6.9	7.0	7.0	6.9	7.0	6.9	
7.3	7.3	7.2	7.2	7.2	7.2	7.2	7.2	7.3	7.2	7.2
7.8	7.8	7.6	7.4	7.4	7.6	7.4	7.4	7.6	7.5	7.4
8.1	8.0	8.0	7.9	7.8	8.0	7.9	7.8	7.9	7.9	7.9
8.6	8.4*	8.2	8.2	8.0	8.2	8.1	8.0	8.2	8.0	
9.0	8.8*	8.3	8.2	8.1	8.3	8.2	8.2	8.4	8.3	

* Precipitate.

Experiment II. The effect of exposure in an atmosphere of CO_2 upon the reaction of broth

Beef infusion broth, prepared and adjusted as outlined in the preceding experiment, was tubed, autoclaved, and treated as follows: (1) Control-beginning. (2) Exposed twenty-four hours in plugged tubes to an atmosphere of CO_2. (3) Control after twenty-four hours.

The results shown in table 6 indicate that direct exposure of broth to CO_2 causes very decided increases in acidity, the amount of increase becoming greater as the alkalinity of the broth

increases. That such a condition is abnormal is, of course, quite obvious, but the experiment serves to indicate that CO_2 may not be ruled out as a factor in causing acidity increases in media upon standing.

TABLE 6

Experiment II

P_H after autoclaving	5.3	5.6	5.8	6.0	6.2	6.5	7.1	7.4	7.6	7.9	8.3	8.4				
P_H after exposure to CO_2 for 24 hours	5.3	5.4	5.5	5.6	5.7	5.8	5.9	6.1	6.2	6.2	6.3	6.4				
P_H (control) after 24 hours	5.3	5.6	5.8	6.0	6.2	6.5	7.1	7.4	7.6	7.9	8.3	8.4				

Experiment III. The effect of exposure of sterilized broth to an atmosphere free from CO_2

Tubes of the medium prepared in the preceding experiment were autoclaved and treated as follows: (1) Control, allowed to stand at room temperature. (2) Placed in a CO_2-free atmosphere. P_H readings were made at the outset, after seven days, and after fourteen days. To obtain atmosphere free from CO_2 air was drawn through a train of Woulff bottles containing concentrated NaOH, 20 per cent $Ba(OH)_2$, and $CaCl_2$ into a large Navy jar containing the tubes of media.

By inspecting table 7, it will be noted at once that practically the same changes in P_H occurred in both sets of tubes. This would seem to dispose of atmospheric CO_2 as a factor operative in causing the increases in acidity so frequently noted in the previous experiments.

TABLE 7

Experiment III

P_H after autoclaving	5.1	5.3	5.8	6.1	6.3	6.6	7.0	7.2	7.6	8.0	8.5	8.7				
P_H after 7 days in atmosphere	5.2	5.5	5.8	6.1	6.4	6.6	7.1	7.4	7.8	8.0	8.3	8.6				
P_H after 7 days in CO_2-free air	5.1	5.4	5.8	6.0	6.4	6.6	7.1	7.4	7.8	8.0	8.3	8.6				
P_H after 14 days in atmosphere	5.0	5.4	5.8	6.1	6.4	6.6	7.0	7.3	7.8	8.0	8.4	8.6				
P_H after 14 days in CO_2-free air	5.0	5.4	5.7	6.0	6.3	6.6	6.9	7.3		7.9	8.3	8.4				

Assuming that the external factors of glassware and atmospheric CO_2 are not sources of change in reaction of broth media it will be necessary next to examine the internal factors, namely, the possibility of chemical changes in the medium itself. The organic

components of broth media are in themselves complex compounds, which in some cases, are relatively unstable and reactive. It has long been noted that in the preparation of media precipitates occur when certain amounts of acid or base are added. In some cases precipitation occurs as soon as the acid or base is added, in other cases autoclaving seems to be required to bring down the material. Kligler (1917) has established certain zones of hydrogen-ion concentration for aqueous solutions of peptone within which precipitation occurs, and has investigated the nature of the precipitates themselves. In the acid range he believes that the material arises largely from protein substances as upon redissolving it gives reactions of proteoses and peptones, whereas in the alkaline range it is made up largely of phosphates. It is rather significant that the ranges of P_H in which we find the greatest change in reaction upon sterilization and standing are those within which precipitation is apt to occur during adjustment of the media.

The rôle of peptone in media is two-fold. It furnishes nitrogenous food in the form of protein split products (peptones, proteoses, peptides, amino acids) and through its property of combining with acids and bases acts as a buffer. According to Rettger, Berman, and Sturges (1916) and Davis (1917) American peptones are lower in albumoses and higher in amino acids than Witte's, some of those examined by the latter having two or three times the content of amino acids.

It seems quite certain that during autoclaving of culture media the higher nitrogenous complexes are hydrolyzed to lower split products. This would be particularly true in media adjusted in the acid or alkaline ranges, inasmuch as acids and bases act as positive catalyzers of a protein hydrolysis. During the splitting of a protein by hydrolysis there occur marked changes in the acidity or alkalinity of the solution in which the change takes place. Sörensen (1912) has reported an experiment in which the digestion of peptone by trypsin was carried out, measurements of hydrogen-ion concentration and determinations of the increase in formol-titrating material being made at intervals. The increase in hydrogen-ion concentration did not stand in relation to

the increase in COOH groups and Sörensen concluded that the increased base-binding power was due to the formation of peptides. T. B. Robertson (1918) has studied rather intensively the changes in hydrogen-ion concentration which take place during the hydrolysis of certain proteins and concludes that the power to bind acids and bases resides in the –COHN– groups, inasmuch as the protein molecule does not contain a sufficient number of terminal –COOH and $-NH_2$ groups to account for its high combining capacity for acids and alkalies. While bound up in the protein molecule these groups do not assist in the neutralization of acids and bases but during hydrolysis the bonds are opened and the binding capacity is increased.

Itano (1916a) has reported an increase in formol-titrating nitrogen in media upon sterilization and has apparently shown that at least a rough proportionality exists between the change in P_H (increase) and the increase in amino acids as measured by the method of Sörensen.

With the view to ascertaining whether or not the changes in P_H found in the experiments described could be correlated with an increase in COOH groups produced through hydrolysis of the peptone or protein of the broth the following experiments were carried out:

Experiment IV. The relationship between P_H changes in media and changes in formol-titrating nitrogen

Five lots of beef infusion broth were adjusted to P_H values ranging from 5.2 to 9.2 and each lot distributed in three 30 cc. portions. The P_H and formol number were determined: (1) before autoclaving; (2) after autoclaving; (3) after seven days standing at room temperature.

Technic of formol titration, Kendall, Day, and Walker (1913): Five cubic centimeters of the broth was diluted with 50 cc. of distilled water and 1 cc. of phenolphthalein (1 per cent alcoholic solution) was added. The material was titrated to a faint pink with N/20 NaOH or N/20 HCl. Five cubic centimeters of neutral formalin were then added and the mixture allowed to stand for thirty minutes after which it was again titrated with

N/20 NaOH. From the last figure, the formol number was obtained.

Formol number (*F. N.*): Milligrams of formol-titrating N per 100 cc. of culture.

The results of the experiment are contained in the following table:

TABLE 8

Experiment IV

BEFORE AUTOCLAVING		AFTER AUTOCLAVING		AFTER SEVEN DAYS	
P_H	F.N.	P_H	F.N.	P_H	F.N.
5.2	40.4	5.3	44.8	5.3	44.8
6.2	42.0	6.3	47.6	6.3	47.6
7.2	42.0	7.2	47.6	7.2	47.6
8.2	43.4	8.0	44.8	8.0	44.8
9.2	43.4	8.8	43.4	8.8	47.6

As a result of autoclaving, slight increases in formol-titrating nitrogen are manifest in every lot of broth excepting that adjusted to P_H 9.2 which was the only flask to show any appreciable change in P_H. The greatest increases in formol number are seen in the lots which showed little or no reaction change. No change in formol-titrating nitrogen occurs during the first seven days following autoclaving except in the 9.2 lot. Here a small increase occurred. From the results of this one experiment it must be concluded that changes in the P_H of broth as a result of autoclaving and standing bear no relationship to changes in formol-titrating nitrogen. The results are at variance with those reported by Itano (1916a) in which decreases in the hydrogen-ion concentration of broth upon autoclaving appeared to be roughly correlated with increases in formol-titrating nitrogen. It perhaps should be noted that fewer changes in P_H were recorded in experiment IV than were apparent in the earlier experiments.

At present, the most logical explanation of acidity increase noted in the various experiments would rest upon the observation of Robertson that as the hydrolysis of a protein proceeds the base-binding capacity of the material increases through the opening up of the –COHN– group of the protein molecule.

SUMMARY AND CONCLUSIONS

1. Broth (beef infusion, beef extract, "bacto-beef") adjusted to P_H values ranging from 5.0 to 9.0 undergoes a change in hydrogen-ion concentration upon autoclaving. This change is most marked in media adjusted in the alkaline range (7.8–9.0), less great in the acid range (5.0–6.2), and is usually inappreciable in the neutral range (6.6–7.4). The maximum change is about 0.4 P_H and in the majority of cases is not over 0.2 P_H.

2. The change is usually an increased acidity (decrease in P_H). Decreases in acidity have been observed in a few instances but these are exceptional.

3. In media of the same composition the reaction changes are not necessarily uniform in different experiments.

4. Autoclaved broth undergoes changes in hydrogen-ion concentration upon standing; the degree of change is not influenced by the environmental temperature within the limits, 10°C. (ice chest) and 37°C. (incubator).

5. The reaction changes upon standing, as in the case of autoclaving, are most noticeable in the alkaline range, less marked in the acid range, and least in the neutral range. Neutral media usually do not change at all upon standing.

6. The change upon standing is almost invariably in the direction of an increase in acidity.

7. Broth adjusted to various P_H levels ranging from 5.0 to 9.0 and exposed in tubes plugged with cotton to an atmosphere of CO_2 for twenty-four hours shows marked alterations in reaction. The change is always an increase in acidity, as would be expected. The greatest change occurs in the alkaline range.

8. Upon allowing broth adjusted to various P_H levels to stand in a CO_2-free atmosphere the same reaction changes were noted as in duplicate lots of broth allowed to stand in the air of the laboratory. The increases in acidity exhibited by broth upon standing do not seem to be due to an absorption of atmospheric CO_2.

9. Reaction changes in media of P_H 5.2 to 9.2 do not appear to stand in relation to changes in formol-titrating nitrogen.

10. The possibility of an increase in acidity of broth through the opening up of –COHN– groups during hydrolysis of the protein constituents suggested by Robertson remains a plausible one.

THE RELATION OF HYDROGEN-ION CONCENTRATION TO THE GROWTH, VIABILITY, AND FERMENTATIVE ACTIVITY OF STREPTOCOCCUS HEMOLYTICUS

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I. THE FINAL HYDROGEN-ION CONCENTRATION PRODUCED BY STREPTOCOCCUS HEMOLYTICUS IN BROTH CONTAINING VARIOUS FERMENTABLE SUBSTANCES

In 1912, Michaelis and Marcora (1912) working with a culture of *Bact. coli* in lactose broth were able to show by means of accurate electrometric measurements, that this organism carries its fermentation of the sugar to a definite level of hydrogen-ion concentration and then ceases its activity. This point is reached regardless of the initial reaction of the medium and can be described as a physiological constant for the particular organism used. This finding was confirmed three years later by W. M. Clark (1915b)[1] who pointed out that the final hydrogen-ion concentration established as a physiological constant by Michaelis and Marcora for a single strain of *Bact. coli* applied to other strains as well. That the hydrogen-ion concentration of the culture, rather than the total acid produced, is the factor limiting activity of the organism seemed evident from the work of Clark. The usefulness of this so-called physiological constant appeared later as the result of the researches of Clark and Lubs (1915) who suggested a method of differentiating the bacteria of the colon-aerogenes group by means of a correlation with gas formation of the final hydrogen-ion concentration produced in glucose broth. In this work was laid the experimental foundation of the methyl

[1] Bibliography is found at the end of the third article in this series, p. 231.

red test in use at the present time. Ayers (1916) in an investigation of the final hydrogen-ion concentration in some 200 cultures of streptococci was able to demonstrate a somewhat higher acidity[2] in cultures of the non-pathogenic than in those of pathogenic species grown upon glucose broth. Later work by Ayers, Johnson, and Davis (1918), as well as by Avery and Cullen (1919a), has led to the suggestion of a rapid presumptive test for the differentiation of bovine and human streptococci based upon differences in the final hydrogen-ion concentration produced in glucose broth. However, as Brown (1920) has pointed out, no single procedure can perhaps serve to differentiate the two varieties absolutely inasmuch as atypical strains are somewhat frequent. Cullen and Chesney (1918), Jones (1920, 1920a), Avery and Cullen (1919b), and Lord and Nye (1919), working with pneumococci of the various types in glucose broth, have found a final hydrogen-ion concentration that is in close agreement with the constant established for the streptococci. This value appears to be the same in all types irrespective of immunological character. The works of Fred and Loomis (1917) upon alfalfa bacteria, of Bunker (1916–1917, 1919) and Davis (1918) upon *Corynebact. diphtheriae*, of Itano (1916a, 1916b) upon *B. subtilis* and certain streptococci, of Cole and Onslow (1916) upon the typhoid group, of Clark (1917) upon *Lactobacillus bulgaricus*, of Waksman and Joffe (1920) upon Actinomycetes, of Ayers and Rupp (1918) upon members of the alkali-forming group, of Wolf and Harris (1917a, 1917b) upon *Clostridium welchii* and *C. sporogenes*, of Gillespie (1916, 1918) on soil organisms, and of Cohen and Clark (1918) upon various organisms are indicative of an attempt on the part of present-day workers to gain a more accurate knowledge of the metabolic activities of micro-organisms through the measurement of changes in the hydrogen-ion concentration brought about in culture media. Determinations of titratable acidity and ammonia, according to Kligler (1916), give an indication of the extent of carbohydrate

[2] The terms *acid* and *acidity* in the present paper refer to true acidity as expressed in terms of P_H, except when the reference is specifically to *titratable acid* or *acidity*.

and protein splitting by bacteria, whereas measurement of the hydrogen-ion concentration in cultures measures the resultant of both these actions.

It seemed advisable at the beginning of the present phase of the investigation to obtain information as to the level of final acidity produced by *Streptococcus hemolyticus* in broth media containing a number of the common fermentable substances employed in the bacteriological laboratory. Accordingly experiment I was carried out.

Methods and technic

Culture. All of the work to be described in the present paper was carried out on one pure strain of *Streptococcus hemolyticus.* This strain, designated as the "H," was originally isolated from the lung in a fatal case of bronchopneumonia complicated by endocarditis, and corresponds to culture number 136 in the series obtained during the investigation of pneumonia in military camps by the Rockefeller Commission. The "H" strain was of high virulence, owing to repeated passage through rabbits in an investigation of experimental streptococcus empyema, and the pleural fluids of such animals, taken at autopsy with sterile precautions (Gay and Stone, 1920), were found to serve as an excellent source of culture material. All pleural fluids were stored in the ice chest as the contained organisms have been found to remain viable under such conditions for a number of weeks. A transplant of 0.2 cc. of the pleuritic exudate was made into 5 cc. of 1 per cent glucose broth and the tube incubated for eighteen hours. As this first generation culture invariably contained a considerable amount of cellular débris, a second subculture was prepared in a similar manner. This second generation served as a source of inoculum in practically all of the experiments to be described. The eighteen-hour incubation period was chosen inasmuch as preliminary tests had shown that rapid growth nearly always obtained in sub-cultures prepared from a parent culture of this age.

Culture media. Beef infusion broth served as the basis of the media employed throughout the work as it is a generally

recognized fact that the pathogenic streptococci develop more luxuriant growth upon this medium than upon broth prepared from beef extract. In some of the experiments "Bacto-beef" (Digestive Ferments Company) was employed instead of beef juice as a base. Growth upon this medium was found to be as luxuriant as upon the usual beef infusion broth. The broth contained 1 per cent peptone (Difco or Parke, Davis, and Company), and 0.5 per cent NaCl. Adjustment to the desired P_H was made according to the method previously described. The limits of P_H, 7.0–7.6, were found to favor luxuriant growth of the organism. The prepared broth was always incubated for twenty-four hours previous to inoculation to insure its sterility.

P_H determinations.[3] These were made by the method described in a former paper using 1 cc. of culture plus 4 cc. of freshly boiled and cooled distilled water. A tube containing the same materials without indicator was always used by the method of superposition to eliminate as far as possible factors of color and turbidity. Determinations carried out in this way permitted readings to within 0.05 P_H in nearly all cases.

Experiment I. The final hydrogen-ion concentration of Streptococcus hemolyticus in broth containing various fermentable substances commonly employed in the bacteriological laboratory; also an attempt to investigate the possibility of an experimental adaptation to a given sugar medium, through repeated transplantation.

Inoculation of 0.4 cc. of an eighteen-hour, second-generation culture was made into 10 cc. lots of beef infusion broth containing the given fermentable material in 1 per cent concentration. Transplants from each tube were made, into sterile lots of media of corresponding composition after twenty-four hours incubation. In this manner five generations were carried. Although the "H" strain had previously been found to produce the characteristic final hydrogen-ion concentration quite con-

[3] The symbol P_H of Sörensen is used throughout to designate the hydrogen ion concentration.

sistently within the first twenty-four hours following incubation, nevertheless in this experiment it was decided to allow a forty-eight-hour incubation period before making P_H determinations to insure the completion of the fermentation.

Reference to table 1 shows that of the several groups of substances tried only the hexoses and disaccharides were fermented by the streptococcus. A wide variation in final P_H is noted. No explanation of these differences is attempted at the present time. Clark (1915b) working with *Bact. coli*, reports lower P_H levels in glucose broth than in lactose broth, while Jones (1920) has described a similar phenomenon in cultures of *Streptococcus hemolyticus* and pneumococci. Similar results are evident in the present experiment. An interesting fact brought out is that plain broth shows an increase in hydrogen-ion concentration. It is also to be noted that in no case in which fermentation did occur was the characteristic final P_H reached in the first generation. This would seem to indicate that in procedures for differentiation based upon final P_H levels, several transfers of the cultures should be made upon the same medium before conclusions as to the final hydrogen-ion concentration are drawn. In nearly all cases the characteristic final value was reached after one transfer.

The fact that plain broth shows an increase in hydrogen-ion concentration when inoculated with the streptococcus would seem to indicate that sufficient muscle sugar is present to permit fermentation to the P_H level indicated. To decide this point, a lot of infusion broth was inoculated with *Bact. coli* to ferment out any free sugar, after which it was filtered, adjusted, and sterilized. Upon inoculation with a culture of *Streptococcus hemolyticus* it was found that the final P_H was the same as that noted in the experiment just described. In this case the initial P_H of the broth was slightly lower, namely, 7.35. A similar result was experienced when sugar-free, bacto-beef broth was tried. In their studies of the metabolism of *Streptococcus pyogenes* and other organisms Kendall and his associates (1912c, 1912a) found increases in titratable acidity in plain broth cultures but carried out no determinations of hydrogen-ion concen-

tration. According to these investigators, the phenomenon may be explained on the basis of a selective action of the organism in question upon that portion of Witte's peptone which Pick (1898) has shown contains a relatively large fraction of a sub-

TABLE 1

Experiment 1

NUMBER	CARBOHYDRATE	P_H (INITIAL)	GENERATION				
			1	2	3	4	5
1	None	7.50	+++ 6.70	++ 6.70	++ 6.70	++ 6.70	
2	Glucose	7.50			+++ 5.10	+++ 4.80	+++ 4.85
3	Fructose	7.50	+++ 5.30	+++ 5.10	+++ 5.10	+++ 5.10	+++ 5.05
4	Mannose	7.50	+++ 5.40	+++ 5.20	+++ 5.25	+++ 5.20	+++ 5.20
5	Galactose	7.50	+++ 5.50	+++ 5.40	+++ 5.40	+++ 5.40	+++ 5.30
6	Xylose	7.50	++ 6.60	++ 6.70	++ 6.70	++ 6.70	
7	Sucrose	7.50	+++ 5.35	+++ 5.20	+++ 5.10	+++ 5.15	+++ 5.10
8	Lactose	7.50	+++ 5.50	+++ 5.50	+++ 5.40	+++ 5.40	+++ 5.40
9	Maltose	7.50	+++ 5.40	+++ 5.30	+++ 5.15	+++ 5.10	+++ 5.15
10	Inulin	7.50	++ 6.60	++ 6.60	++ 6.70	++ 6.70	++ 6.70
11	Glycerol	7.50	++ 6.60	++ 6.70	++ 6.70	++ 6.70	++ 6.50
12	Mannite	7.50	++ 6.60	++ 6.60	++ 6.70	++ 6.70	++ 6.70

stance reacting typically like a carbohydrate. Although the peptone used in the present experiments was not Witte's it seems entirely possible that American peptones such as the one used here (Parke, Davis and Company) might contain a similar carbohydrate substance. The fact that a definite increase in hydrogen-ion concentration has always been observed in the sugar-free broth employed surely would lend support to such a supposition.

II. THE INFLUENCE OF VARYING AMOUNTS OF GLUCOSE AND BUFFER SALTS UPON THE FINAL HYDROGEN-ION CONCENTRATION OF STREPTOCOCCUS HEMOLYTICUS

It has long been recognized that the acidity produced by certain organisms in culture media results from the elaboration of acid substances through a fermentation of material, mainly of carbohydrate nature. With the introduction of accurate methods of evaluating the acidity produced in bacterial fermentations through a determination of the concentration of the hydrogen-ions, it became necessary to investigate the factors which may be operative in the production of a limiting or final hydrogen-ion concentration. Thus, Clark and Lubs (1915) in their work on the differentiation of the bacteria of the colon-aerogenes family, used media containing amounts of glucose varying from 0 to 0.5 per cent and demonstrated that by increasing the concentration of the sugar up to a certain point a greater final acidity resulted. If sufficient sugar was present for the limiting acidity to be produced, no alkaline reversion occurred in their cultures. Browne (1914), using cultures of *Bact. coli* in lactose-broth, found that acid production was less marked in media containing under 1 per cent sugar but that the use of amounts over 1 per cent resulted in no increase. Browne titrated his cultures with N/20 NaOH but failed to make determinations of the final hydrogen-ion concentration. Avery and Cullen (1919b) found that pneumococci were able to reduce the P_H of glucose-broth from 7.50 to 5.10 provided 0.4 per cent of the sugar was present. Increasing concentrations of glucose up to 4 per cent showed no change in

final P_H. In the work of the same investigators (1919a) upon Streptococci of human and bovine origin it was shown that the same final P_H is reached in broth containing 0.5, 1, or 1.5 per cent of glucose. Sekiguchi (1917) found the highest production of acid by streptococci with 0.5 to 2 per cent of glucose. Amounts of sugar over 5 per cent caused reduction in acid formation though growth was not hindered. H. Jones (1920) has recently found that a number of organisms are able to produce their characteristic final hydrogen-ion concentration provided 0.2 per cent or more glucose be present in the medium. He failed to state the initial P_H of the medium which factor has an important bearing on the minimum concentration of a sugar needed for production of the final acidity by any given organism. The effect of varying amounts of xylose upon the production of volatile acid by xylose fermenting organisms has been studied by Fred, Peterson, and Davenport (1919) who found that 2 per cent of the sugar gave the maximum production of acid. The presence in the culture medium of substances which through their buffer effect have the power of neutralizing some of the acid as it is produced is of interest and importance in this connection.

Henderson and Webster (1907) in 1907 suggested the use of phosphates to preserve neutrality in media during the growth of acid- or alkali-forming organisms, and Clark (1915a) has more recently pointed out in considerable detail the great importance of properly buffered media in bacteriological work. Using lots of broth containing different buffers, Clark (1915b) showed that *Bact. coli* produces somewhat lower levels of P_H in the more highly buffered media.

Kligler (1916) working with cultures of *Bact. cloacae*, *Bact. aerogenes*, and *Bact. coli* studied the final P_H as influenced by different concentrations of peptone, Na_2HPO_4, and glucose. The concentration of peptone was found to influence the utilization of glucose by the organisms in such a way as to result in a lower final P_H with a low peptone concentration in the medium. In some cases the presence of buffer allowed all of the sugar to be used up with a subsequent rise of P_H thus indicating that an

alkaline phase had been initiated through the splitting of peptone. The presence of buffer, according to Kligler, keeps the hydrogen-ion concentration below the lethal point and thus allows the organism to continue its activity over a longer period. As a result of this regulatory power the amount of glucose which may be used will vary, within limits, with the relative amount of buffer material present. Bronfenbrenner and Schlesinger (1918) working with *Bact. coli* have tried similar experiments by noting the effects of varying amounts of lactose, peptone, and buffer salts upon gas formation and final P_H. After trying some 294 combinations, these investigators concluded that with any given concentration of carbohydrate the amount of free acid depends upon the concentration of buffer in the medium. As the amount of peptone increases, the per cent of sugar attacked is smaller and lower hydrogen-ion concentrations result. The necessity of carefully controlling the composition of media employed in fermentation experiments is emphasized.

From the foregoing review the following facts seem to have been well established:

1. In any given medium a definite concentration of sugar must be present if the organism in question is to produce its characteristic final hydrogen-ion concentration.

2. This minimum concentration of sugar will depend upon the concentration of buffer salts present, as well as upon the concentration of peptone in the medium.

3. In making estimations of this minimum concentration of sugar required for the production of the final hydrogen-ion concentration the quantity of buffer should be known as well as the initial P_H of the culture medium.

4. With increasing concentrations of buffer salts there is an increased neutralizing power which delays the production of the final acidity level, thus allowing the organism more time for fermentation.

Experiment II. The effect of varying concentrations of glucose upon the final hydrogen-ion concentration of Streptococcus hemolyticus

Ten cubic centimeter amounts of beef infusion broth containing concentrations of glucose varying from 0.10 to 1 per cent were inoculated with 0.4 cc. of an eighteen-hour culture of *Streptococcus hemolyticus* in 1 per cent glucose broth and incubated for three days to insure the completion of the fermentation. P_H readings were then made. The results are shown in table 2.

TABLE 2
Experiment II

NUMBER	MEDIUM	P_H (INITIAL)	P_H (FINAL) IN GLUCOSE (PER CENT)					
			0	0.1	0.2	0.3	0.5	1.0
1	Beef infusion broth	6.90		5.60	5.00	5.10	5.05	5.10
2	Beef infusion broth (sugar free)	7.35	6.70	6.05	5.60	5.10	5.15	5.00

In (1) which was adjusted to an initial P_H of 6.9 the final P_H was attained in a glucose concentration of 0.2 per cent, whereas in (2) which was adjusted to an initial P_H of 7.35 the final value was not shown in the 0.2 per cent glucose but did appear in the 0.3 per cent tube. As would be expected the minimum concentration of glucose needed to give the characteristic final P_H is dependent upon the initial P_H of the broth. Amounts of glucose over this minimum concentration have no further effect upon the level of the final hydrogen-ion concentration.

Experiment III. The influence of a buffer salt upon the final hydrogen-ion concentration of Streptococcus hemolyticus in broth containing varying concentrations of glucose

Bacto beef broth was adjusted and distributed in twelve lots in flasks. After autoclaving, the requisite amounts of glucose and di-potassium phosphate, K_2HPO_4, were added in the form of sterile 10 per cent solutions bringing the total volume of material in each flask to 25 cc. Following twenty-four

hours incubation to insure sterility each flask was inoculated with 1.25 cc. of an active twenty-two-hour culture. Determinations of P_H and "reaction" were made after an incubation period of four days. The "reaction" was determined by titrating 5 cc. of culture with N/50 NaOH, using neutral red as an indicator and calculating the number of cubic centimeters of N/1 NaOH needed to neutralize the acid in 100 cc. of culture. Table 3 contains the results of the experiment.

TABLE 3

Experiment III

NUMBER	GLUCOSE	K_2HPO_4	P_H (INITIAL)	P_H (FINAL)	"REACTION"*
	per cent	*per cent*			
1	0.3	0	6.90	5.10	0.72
2	0.3	0.2	6.90	5.05	1.41
3	0.3	0.5	7.20	5.20	2.13
4	0.3	1.0	7.20	6.30	2.43
5	0.5	0	6.75	5.20	0.70
6	0.5	0.2	6.70	5.00	1.54
7	0.5	0.5	7.20	5.05	2.18
8	0.5	1.0	7.20	6.20	2.16
9	1.0	0	6.90	5.15	0.81
10	1.0	0.2	6.90	5.00	1.56
11	1.0	0.5	7.20	4.90	3.36
12	1.0	1.0	7.20	5.20	4.73

* Cubic centimeters of N/1 NaOH required to neutralize 100 cc. of culture.

As will be seen by referring to table 3 the final P_H characteristic of the streptococcus is not reached in the media containing 0.3 per cent and 0.5 per cent glucose plus 1 per cent phosphate (numbers 4 and 8 in table). These concentrations of glucose are apparently not sufficiently great to allow the formation of enough acid to bring the culture to the characteristic level, whereas in the case of the 1 per cent glucose plus 1 per cent phosphate a characteristic final P_H is reached. Correlated with these facts are the differences in titratable acid as shown in the last column of the above table. It is an interesting fact that virtually the same final P_H is shown in the greater number of the

above cases and yet the total quantities of actual acid, as shown by titration, are widely different. No better illustration of the efficiency of a buffer could be offered. Very obviously the utilization of glucose is here closely related to the concentration of buffer present. A further fact, of interest and importance, is that the final hydrogen-ion concentration rather than the total acid produced is the factor which limits the fermentative activities of the organism.

Experiment IV. The influence of horse serum in glucose broth upon the final P_H *of Streptococcus hemolyticus*

Ten cubic centimeter lots of beef infusion broth (sugar-free) containing varying amounts of glucose and horse serum were prepared and inoculated with 0.4 cc. of an eighteen-hour culture. After an incubation of three days P_H determinations were made. The results of (2) in experiment II are inserted in table 4 for purposes of comparison.

TABLE 4
Experiment IV

NUMBER	HORSE-SERUM	P_H (INITIAL)	P_H (FINAL) IN GLUCOSE (PER CENT)					
			0	0.1	0.2	0.3	0.5	1.0
	per cent							
1	5.0	7.40	6.80	6.70	6.10	5.05	5.00	5.00
2	10.0	7.60	6.80	6.60	5.90	5.15	5.00	5.10
2 (exp. II)	None	7.35	6.70	6.05	5.60	5.10	5.15	5.00

As in experiment II it is to be noted that 0.3 per cent glucose is the minimum concentration which will permit the attainment of the characteristic final P_H. The greatest differences in P_H between the media containing horse serum and (2) of experiment II are seen in the tubes containing 0.1 per cent and 0.2 per cent glucose. It seems possible that in these cases the horse serum prevents the increase in acidity of the medium to a small extent through its action as a buffer. In those tubes containing sufficient glucose for the production of the final P_H characteristic of the organism no differences in the level of this final value are seen. That we do have a decided difference in the rates of acid production will be shown in a later experiment.

Experiment V. The buffer action of horse serum in broth

To investigate further the buffer effect of horse serum titration curves of broth containing 1 per cent glucose, 1 per cent glucose plus 5 per cent horse serum, and 1 per cent glucose plus

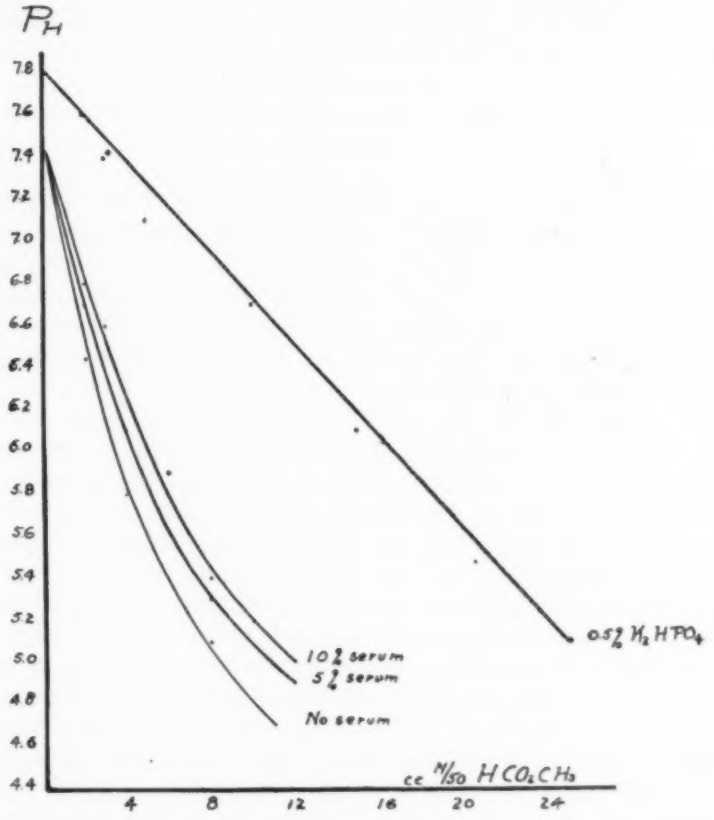

FIG. 1. EXPERIMENT V

10 per cent horse serum were plotted after the following procedure had been carried out: To 10 cc. portions of the three types of broth mentioned above, amounts of N/50 acetic acid varying from 1 to 12 cc. were added and the P_H taken. The curves were

plotted using the cubic centimeter of acid as abscissae and the P_H readings as ordinates. Reference to the curves (fig. 1) will show that horse serum in these concentrations exerts a slight but distinct buffer effect. The 10 per cent series does not show a much greater buffer action than the 5 per cent series however, and the effect in no case is anything like that noted in the case of K_2HPO_4 (experiment III).

III. THE RATE OF ACIDITY FORMATION IN CULTURES OF STREPTOCOCCUS HEMOLYTICUS

Considerable work by a number of investigators has demonstrated that the life cycle of a given organism, as measured by the number of viable cells present at various intervals following inoculation, may be separated into very definite periods. Thus, Chesney (1916) has suggested a division into four phases: (1) latent period or lag, (2) maximum growth period, (3) stationary period, (4) period of decline.

No sharp dividing lines may be drawn between the periods, and their duration will vary in the case of the same organism with such factors as the amount of inoculum, age of parent culture, and initial reaction of the medium. Buchanan (1918) described seven periods in the life of an organism: (1) initial stationary phase; (2) lag phase when growth proceeds at a slowly accelerating rate; (3) maximum or logarithmic period in which the rate of increase in numbers is constant; (4) period of negative growth acceleration, the organisms are increasing at a decreasing rate; (5) maximum stationary period; no increase in numbers; (6) period of accelerated death, decrease in taking place at an increasing rate; (7) logarithmic death phase; death is occurring at a constant rate.

With the development of procedures for the mathematical analysis of the several phases (Buchner, Longard, and Riedlin (1887), Buchanan (1918), Slator (1917), Ledingham and Penfold (1914) has come the possibility of more definite knowledge concerning the growth activities of organisms.

A search through the literature reveals the fact that the latent period or lag phase has received the bulk of the attention of workers in this field. Müller (1896) perhaps was the first to recognize the phenomenon while working with cultures of *Bact. typhosum* at temperatures simulating febrile conditions. The duration of lag was found by him to vary with the age of the culture used for seeding, being shorter for young than for older cultures. He believed the phenomenon to be the result of an alteration of the cells sustained upon transplantation to a new medium, the duration of lag representing the time required for the organisms to recover from the injury. Rahn (1906), working with *Ps. fluorescens*, studied the influence upon lag of the amount of inoculum and concluded that the larger the number of organisms used for seeding, the shorter the lag. Penfold (1914) later demonstrated that this effect held, up to a certain limit, beyond which an increase in the amount of inoculum exerted no influence upon the duration of the lag period. In case of small inocula, however, Penfold showed that a diminution in amount of seed invariably caused a lengthening of lag. He found that older cultures caused lengthening of lag only up to a certain point, for example, a four-day culture gave the same duration of lag as a twelve-day culture in the case of *Bact. coli*. Barber (1908) working with single cells (*Bact. coli*) was the first to show that under proper conditions lag may be eliminated. He used rapidly dividing cells which were accustomed to the medium employed and was able to find no evidence of inhibition upon transplantation. This observation has received substantiation at the hands of Penfold (1914), Chesney (1916), and Salter (1919), all of whom worked with *Bact. coli*. Coplans (1909) also states that with *Bact. coli*, there is ordinarily no absolute lag upon transplantation to a favorable medium. New milk ordinarily possesses inhibitory properties but this investigator found that heating momentarily to 100°C. caused a disappearance of this special inhibitory quality. Salter (1919) found also that the age of the parent culture exerted a considerable influence upon the duration of lag, thus confirming the observations of previous investigators. Lane-Claypon (1909) has studied the

rate of growth of organisms as affected by different temperatures and has demonstrated a conformity of her curves with the Van't Hoff-Arrhenius law within certain limits.

The various other phases in the life of a culture have been investigated to a less extent but from the work of Buchanan (1918) and Ledingham and Penfold (1914) it seems probable that growth is a discontinuous process in the sense that development of a given organism is dependent upon different laws in the successive phases of the life of the culture.

An illustrative curve follows:

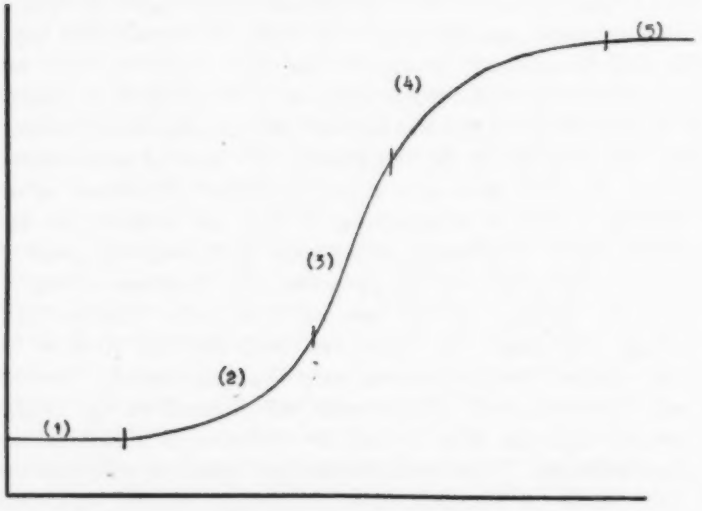

FIG. 2. ILLUSTRATIVE CURVE OF ACID FORMATION BY STREPTOCOCCUS HEMOLYTICUS IN GLUCOSE BROTH

(1) Initial stationary period.
(2) Lag period. Acid formed at a slowly increasing rate.
(3) Maximum period. Acid formed at a constant, maximum rate; curve is an oblique, straight line.
(4) Period of negative acceleration. Acid formed at a decreasing rate.
(5) Maximum stationary period. Final P_H has been attained; curve is a straight line parallel to the abscissa.

If broth containing glucose be inoculated with an actively growing culture of *Streptococcus hemolyticus* and incubated, there ensue changes in the hydrogen-ion concentration of the medium culminating in the establishment of a limiting or final reaction. A study of these changes, as measured at regular intervals, indicates that the course is a perfectly definite one capable of being separated into the following characteristic phases: (1) Initial stationary period, no change in reaction; (2) latent or lag period, acid formation at a slowly increasing rate; (3) maximum period, acid formation at a constant rate; curve an oblique straight line; (4) period of negative acceleration, acid formation at a decreasing rate; (5) maximum stationary period, final P_H reached, curve a straight line parallel to the abscissa.

It will be noted that this sub-division of the course of reaction change corresponds with Buchanan's life phases of a bacterial culture based upon numerical determinations of viable organisms, with the exception that his two final periods, representing a decrease in number, cannot, of necessity, apply to an acid curve such as is characteristic for the streptococcus. The work of Cullen and Chesney (1918) on pneumococci has shown a close relationship between growth-rate and speed of acid production in plain broth, and accordingly these observers have concluded that acid formation is to be considered as an active metabolic process, closely associated with the growth activities of the organism. In examining the curves of Cullen and Chesney one is struck by the close parallelism that exists between the various phases in the life of the pneumococcus, as measured by numbers of viable cells on the one hand, and by acid formation on the other hand. As might be expected, a rise in the growth curve always preceded slightly a rise in acidity. Lord and Nye (1919) have reported results of similar nature on pneumococci grown in glucose broth. During the first 12 hours of their experiment the medium was found to change in reaction from P_H 7.65 to 5.25. Up to this point, a rapid increase in the number of cells was evident, but during the subsequent acidification to the final P_H, 5.15, a rapid decrease in viable organisms was apparent.

In both of these investigations it is evident that the maximum changes in acid formation take place simultaneously with a rapid development and multiplication of the bacteria and thus show a conformity with the conception of Slator (1916) that "Chemical reactions brought about by microorganisms usually proceed under conditions where development of the organism and changes in the composition of the nutrient medium take place simultaneously." H. M. Jones (1920a) however, has recently obtained results which contradict the work of Cullen and Chesney, and Lord and Nye. Using cultures of pneumococci in glucose broth this investigator has shown that the growth curve rises sharply at the fourth to fifth hour while the onset of the maximum period of acid formation is delayed until the twelfth hour. Examination of the curves of this experiment shows the maximum period of growth to be associated with but a slight alteration in the reaction of the medium (7.4–7.0), whereas, the interval of acid formation at a maximum rate corresponds with the period of growth at a decreasing rate. This finding corresponds more or less closely to the observations of Cohen and Clark (1918) upon *Bact. coli* in glucose broth cultures. The growth curve was found to rise five hours previous to the onset of the maximum period of acid production, and, as in the experiments of Jones, the maximum period of acid formation was found to be coincident with the period of growth at a decreasing rate. At the point where strong symptoms of growth inhibition appeared, the P_H was found to correspond to the region at which acetic acid had been previously shown to check growth (5.5–5.7). The fermentative activity, however, was not seriously checked until the culture approached the region in which HCl had been found to inhibit growth (4.6–5.0). From a consideration of these findings it will appear, in the cases of *Bact. coli* and the pneumococcus at least, that the hydrogen-ion concentration may exert independent effects upon growth, on the one hand, and upon acid formation on the other, so that in experiments designed to follow the acid production of organisms in carbohydrate media it will be unsafe to assume that maximum changes in reaction parallel maximum rates of multiplication of bacterial cells.

Clark (1915b), working with *Bact. coli*, was perhaps the first to follow reaction changes in bacterial cultures by means of determinations of hydrogen-ion concentration. No change in P_H was noted under a period of ten hours in his experiment. Itano (1916a) followed the changes in acidity in cultures of *B. subtilis* and noted in certain media of unfavorable initial P_H that an "automatic adjustment" toward a more favorable reaction occurred during incubation. Working with *Clostridium perfringens* (*C. welchii*) and *C. sporogenes* (Metchnikoff), Wolf and Harris (1917b) found that curves of acidity change followed closely those of amino acid formation and gas production. Avery and Cullen (1919b) used media of different initial P_H with pneumococci and demonstrated that after completion of lag, growth, as evidenced by the rate of reaction change, proceeded at about equal speeds. Neither the final P_H nor the rate of acid formation was affected by the use of various available mono- or di-saccharides. The maximum period was found to lie between the fourth and eighth hours following seeding. Bunker (1919) noted an initial acidity rise followed by alkaline reversion in cultures of *Corynebact. diphtheriae* and apparently has shown that toxin production is closely associated with this phenomenon, as no toxin could be demonstrated in cultures which failed to exhibit an alkaline reversion. In a study of the logarithmic or maximum period in cultures of several organisms by Cohen and Clark (1918) it was observed that bacteria may multiply rapidly for a time in media varying considerably in initial reaction. The maximum period of growth in the case of *Bact. coli* fell between the fifth and tenth hours. Schoenholz and Meyer (1919), in their work on *Bact. typhosum*, have reported changes in the growth curve through the influence of hydrogen-ion concentration. Thus they found that growth at a maximum rate set in five hours following incubation, if the initial P_H of the medium was 7.0. At lower and higher levels lag was of longer duration.

Avery and Cullen (1919a), using streptococci of human and bovine origin, found the greatest increase in acidity between the seventh and twelfth hours, using eighteen-hour cultures as

sources of inocula. H. Jones (1920) has recently observed that in the case of pathogenic streptococci the age of the parent culture employed may exert a considerable influence upon the abundance of growth in sub-cultures which may, in turn, be reflected in the final P_H values. He also observed that cultures which were placed under conditions which tended to delay growth failed to show the characteristic final P_H. The statement frequently made that the final P_H of an organism is eventually reached, provided the culture exhibits growth, obviously can not apply to a delicate organism such as the streptococcus. Thro (1915) called attention to the same fact in his observation that with streptococci variations in luxuriance of growth were associated with differences in the quantities of acid substances produced.

Slator (1916) has devised an ingenious method for measuring the rate of growth of a lactic acid-forming organism through an indirect application of the titration values obtained at definite intervals throughout the course of the experiment. Using the formula suggested in a previous work (1917) he was able to show close agreement in the values of the constant, k, in different determinations. The possibility of simultaneous acid and alkaline fermentations in cultures of certain organisms has been emphasized by Ayers and Rupp (1918) who state that such actions may complicate and decrease the value of acidity determinations in certain cases. Methods of measuring both fermentations have been suggested by these investigators.

From the foregoing review it would appear that a study of the progress of reaction changes in cultures of *Streptococcus hemolyticus*, in order to furnish data of value, must of necessity entail an investigation of a number of interacting factors. Accordingly, experiments were planned to study the rate of acid formation as influenced by the following: (1) Amount of inoculum; (2) age of parent culture; (3) presence of a body fluid, horse serum, (4) initial reaction of medium.

Experiment VI. The influence of the amount of inoculum upon the rate of acid formation in glucose broth

Twenty cubic centimeters of 1 per cent glucose broth, P_H 7.10, were inoculated with varying amounts of an active, eighteen hour culture of *Streptococcus hemolyticus* in 1 per cent glucose broth and incubated. At two-hour intervals P_H determinations were made on 1 cc. samples removed from the cultures with aseptic precautions. All cultures remained uncontaminated throughout the entire period of the experiment. The results of the experiment are to be found in table 5 and figure 3.

TABLE 5

(*Experiment VI*)

NUMBER	INOCULUM	DURATION STATIONARY PERIOD	DURATION LAG PERIOD	ONSET OF MAXIMUM PERIOD	DURATION MAXIMUM PERIOD	P_H LOWERING (MAXIMUM PERIOD)	
						Total	Per hour
	cc.	*hours*	*hours*				
1	0.2	8	6+				
2	0.4	2	8	10	2	0.8	0.4
3	0.8	None	8	8	4	1.35	0.34
4	2.0	None	6	6	2	1.40	0.70
5	4.0	None	4	4	6	1.45	0.24

Reference to the curves (fig. 3) shows that the rates of acid formation are at least roughly proportional to the quantities of inoculum used. It is interesting to find that the hourly rate (table 5) during the maximum period is least in the case of (5) notwithstanding the fact that this contained the largest inoculum. In other words, cultures (4), (3), and (2) though showing more prolonged lag periods than (5), are able to proceed with acid formation at more rapid rates, once the maximum period is initated. No P_H determinations were made within the initial two-hour interval, hence it is not possible to assume that any of the cultures showed an entire absence of the stationary period. In (4) and (5), however, the stationary period, if present, was probably of very short duration.

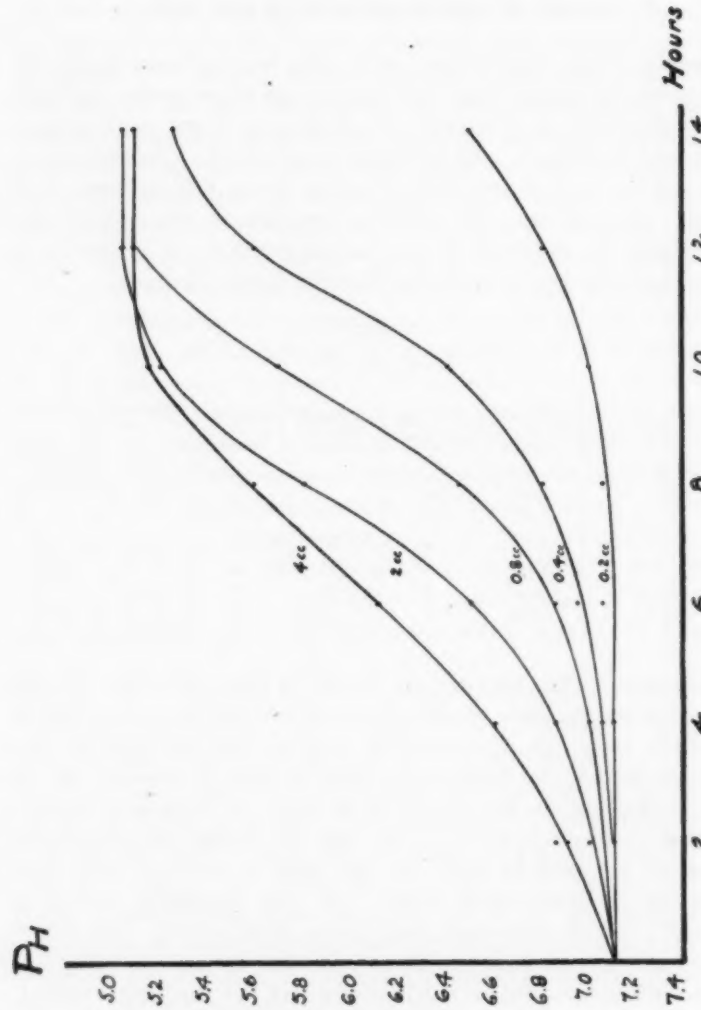

FIG. 3. EXPERIMENT VI. THE INFLUENCE OF THE AMOUNT OF INOCULUM UPON THE RATE OF ACIDITY FORMATION

Experiment VII. The relation of the age of parent culture to the rate of acid formation in glucose broth

Four cultures of *Streptococcus hemolyticus* were made in the usual manner at intervals of six hours. After eighteen hours incubation sub-cultures were made and these second-generation cultures incubated. The schedule was so arranged that at the time of the final inoculation into the medium of the experiment (20 cc. portions of 1 per cent glucose broth) organisms would be taken from parent cultures of six, twelve, eighteen and twenty-four-hours age. One hour previous to the final seeding counts of each parent culture were made by the method of Wright in order that each tube of broth to be used in the experiment might receive approximately the same number of organisms. The inoculum was based upon the proportion, 0.8 cc. of a twenty-four-hour culture per 20 cc. of broth.

Bacterial counts

NUMBER	AGE	ORGANISMS	INOCULUM
	hours	*millions per cu. mm.*	*cc.*
1	6	248	7.40
2	12	1088	1.68
3	18	1548	1.48
4	24	2282	0.80 (basis)

Examination of the curves (fig. 4) shows that (2) (from twelve hour culture) reaches the characteristic final P_H earliest, then come in order the tubes from the six-, eighteen- and twenty-four-hour parent cultures. The onset of the maximum period is seen to follow the same order. As might be expected, the differences are shown almost entirely in the duration of the lag and stationary periods of the four cultures. It is a fact of interest and importance that the rates of acid formation during the maximum period (table 6) were practically equal in the four cases.

From a consideration of the work of various investigators upon the life phases of an organism the results obtained here are not unexpected. It has been repeatedly demonstrated that the maximum rate of acid formation in glucose broth occurs between

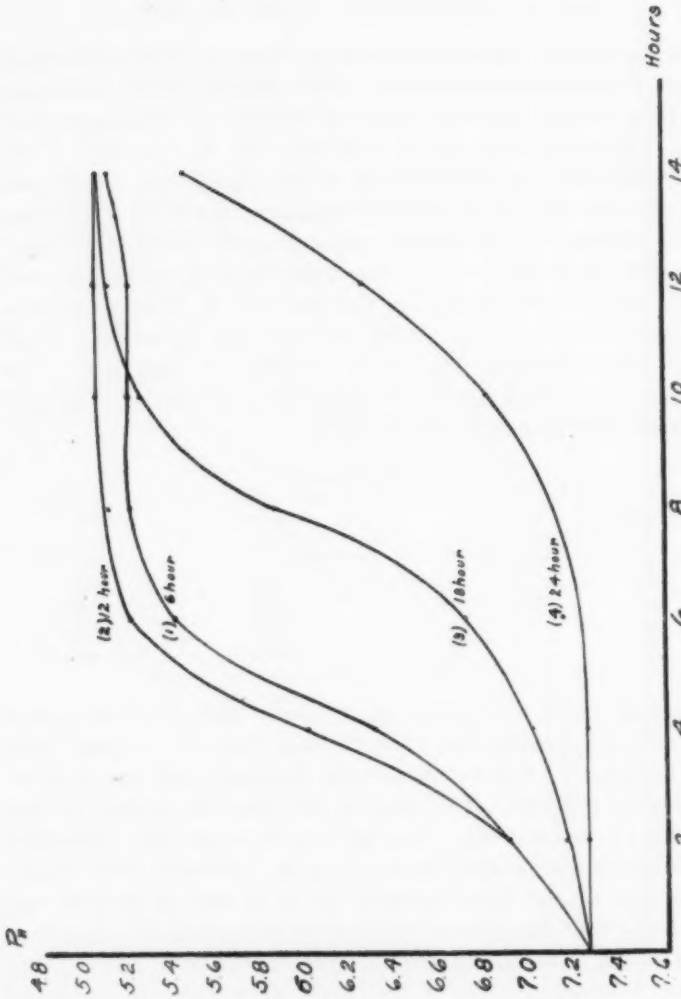

FIG. 4. EXPERIMENT VII. THE INFLUENCE OF THE AGE OF PARENT CULTURE UPON THE RATE OF ACIDITY FORMATION IN SUBCULTURES

the sixth and twelfth hours of incubation, provided the inoculum be taken from an eighteen-hour parent culture. If it be assumed that during this interval the organisms are growing rapidly and that their metabolic activities are at a maximum it would be anticipated that transplantation of organisms during this period to a favorable medium would result in resumption of growth and metabolism with a minimum of lag. The close parallelism in the curves of (1) and (2) bears out this supposition. That the organisms decrease progressively in vitality with the lengthening of their period of contact with the products of their own metabolism is brought out in the curves of (3) and (4).

TABLE 6
Experiment VII

NUMBER	AGE OF PARENT CULTURE	DURATION STATIONARY PERIOD	DURATION LAG PERIOD	ONSET OF MAXIMUM PERIOD	DURATION MAXIMUM PERIOD	PH LOWERING (MAXIMUM PERIOD)	
						Total	Per hour
	hours	*hours*	*hours*		*hours*		
1	6	0	4	4	2	0.90	0.45
2	12	0	2	2	2	0.90	0.45
3	18	0	6	6	2	0.85	0.425
4	24	4	8	12	2 (?)	0.80	0.40

Here are seen more prolonged lag periods, indicating that the organisms required more time to recover from the injury sustained in the previous environment. The injury, however, appears to be only temporary for in all cases acid production is seen to proceed at practically the same rate following the onset of the maximum period. The entire absence of lag in acid production has never been observed with the streptococcus.

Experiment VIII. The rates of acid formation of Streptococcus hemolyticus in glucose broth and in glucose-serum broth

Forty cubic centimeter portions of infusion broth (initial P_H 7.20) containing (1) 1 per cent glucose, and (2) 1 per cent glucose plus 5 per cent horse serum were inoculated with 1.6 cc. of an eighteen-hour glucose broth culture and incubated at 37°. Determinations of hydrogen-ion concentration were made at

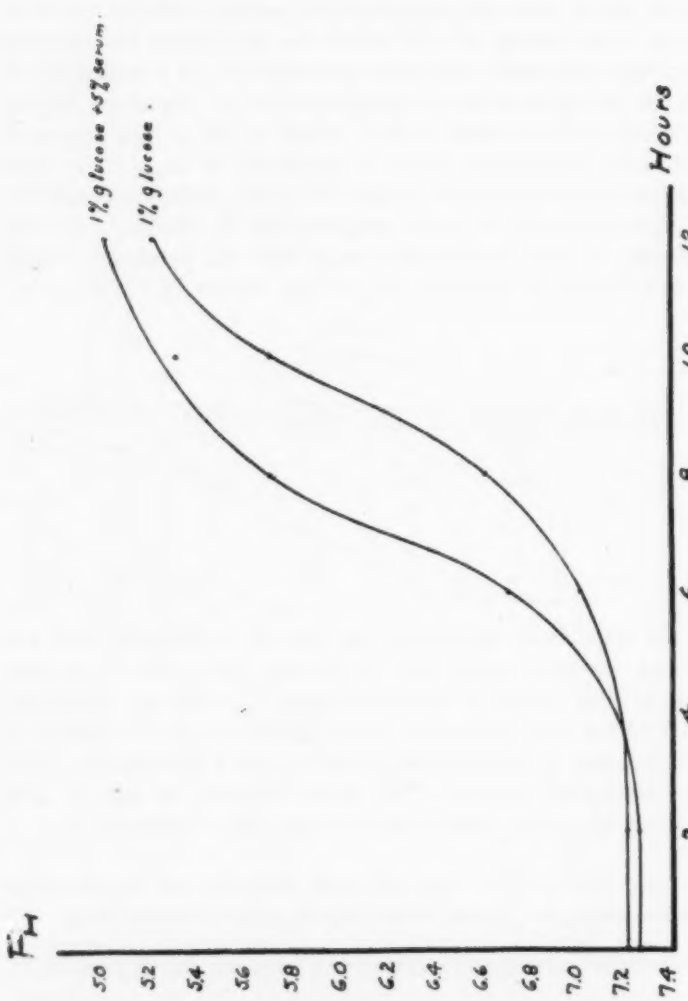

FIG. 5. EXPERIMENT VIII. THE RATE OF ACIDITY FORMATION IN GLUCOSE BROTH AND GLUCOSE-SERUM BROTH

the outset and at two-hour intervals by removing aseptically 2 cc. of material from the flasks. The experiment continued through twelve hours, at the conclusion of which period both cultures had reached their characteristic final level of P_H.

The outstanding fact here, as may readily be seen by reference to the curves (fig. 5), is a more rapid attainment of high levels of acidity on the part of the culture containing horse serum. Though a stationary period of two hours is noted in each, the lag in the glucose culture is of two hours longer duration than in the glucose-serum culture. A close parallelism in rates is seen during the maximum period.

It would seem logical to expect that the differences manifest in the above experiment would be closely correlated with the rates of increase in numbers of cells in the two cultures; in other words, multiplication at a maximum rate would be initiated earlier in the serum-glucose medium. It is a well recognized fact that we have at our disposal no very satisfactory method of enumerating viable streptococci. The method of Wright, though useful in the standardization of bacterial vaccines, gives only approximate results, and moreover, furnishes values which represent the total organisms, viable and nonviable, present in a culture. On the other hand, the method of plating dilutions of a culture which is recognized as valuable in numerical determinations of such organisms as *Bact. coli* and *Bact. typhosum*, is not adequate for enumerations of streptococci owing to the fact that single colonies upon the plate almost invariably represent streptococcal chains of varying length. Moreover, there arises a possibility of the breaking up of coccal chains through the mechanical disturbance occasioned in preparing dilutions of the culture.

Though the inadequacies of these two procedures were recognized it was nevertheless considered advisable to repeat experiment VIII supplementing the P_H determinations at two-hour intervals with estimations of the number of viable organisms through the medium of plate counts.

Experiment IX. The relationship between the rates of acid formation and growth of Streptococcus hemolyticus in glucose broth and in glucose-serum broth

Forty cubic centimeter portions of 1 per cent glucose broth and 1 per cent glucose-5 per cent horse-serum broth were prepared and incubated to insure sterility. Inoculations were made from an eighteen-hour, second-generation culture in 1 per cent glucose broth into the two lots of media. P_H determinations and plating of dilutions were carried out every two hours. The experiment continued through twelve hours.

Technic of plating. 1.8 cc. of plain broth were used as diluting fluid throughout. 0.2 cc. of culture was transferred into this amount of broth and the fluids mixed by carefully drawing up and down in the pipette, after which 0.2 cc. of this dilution were added to 1.8 cc. of broth, etc. until all the dilutions required had been made. Especial care was taken to avoid agitation of the material during the preparation of the dilutions. Nutrient agar containing 10 per cent of defibrinated rabbit's blood was used as a plating medium.

Table 7 contains the results of the experiment.

TABLE 7
Experiment IX

HOURS	GLUCOSE		GLUCOSE SERUM	
	P_H	Counts*	P_H	Counts*
0	7.65	1.68	7.65	1.68
2	7.65	0.38	7.65	40.30
4	7.60	158.00	7.20	140.00
6	7.50	1,498.00	5.90	76,000.00
8	6.80	3,243.00	5.15	713,600.00
10	5.90	250,000.00	5.05	Infinite
12	5.50	Infinite	4.90	1,040,000.00

* Counts are expressed in millions per cubic millimeter.

Attempts to construct growth curves by plotting the logarithms of counts against time brought out certain irregularities which made impossible the formation of smooth curves. Con-

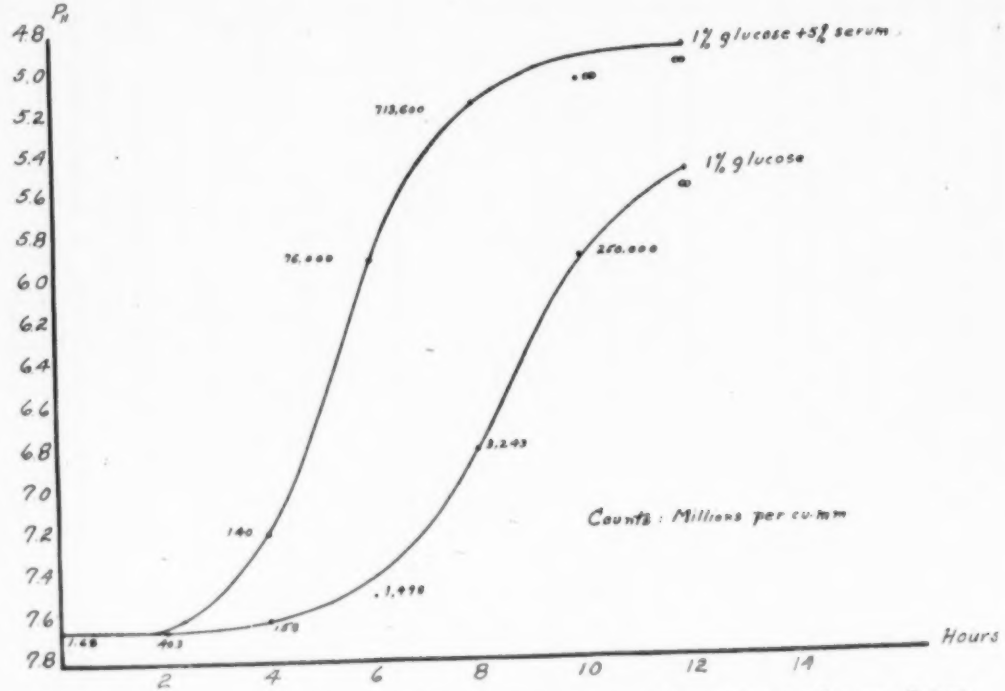

FIG. 6. EXPERIMENT IX. THE RATE OF GROWTH AND ACIDITY FORMATION IN GLUCOSE BROTH AND GLUCOSE-SERUM BROTH

sequently this procedure was abandoned. Curves of acid formation are shown in figure 6. At each point on the curves the number of organisms, expressed as millions per cubic millimeter, is shown. Examination of figure 6 shows that the two curves are analogous to those of experiment VIII (fig. 5), though the lag registered by the glucose-serum culture is of less duration. The numbers of viable organisms as shown by plate counts bear out the assumption that the earlier rise in acidity in a glucose-serum broth is associated with a corresponding period of multiplication at a rapid rate.

Experiment X. The relation of the initial P_H of glucose-broth to the rate of acid formation by Streptococcus hemolyticus

Beef infusion broth was adjusted to various P_H levels, divided into six portions, and sterilized in the usual manner. After adding the proper amount of glucose, the tubes, containing 20

TABLE 8

Experiment X

NUMBER	P_H (INITIAL)	DURATION OF STATIONARY PERIOD	DURATION OF LAG PERIOD	ONSET OF MAXIMUM PERIOD	DURATION OF MAXIMUM PERIOD	P_H LOWERING (MAXIMUM PERIOD)	
						Total	Per hour
1	5.20			No growth			
2	6.20	10		Not reached in 14 hours			
3	7.00	2	4	6th hour	2	0.85	0.425
4	7.50	2	2	4th hour	4	1.70	0.425
5	8.10	2	4	6th hour	2	1.25	0.625
6	8.65	2	?	Not reached	?		

cc. of medium each, were incubated to insure sterility. The inoculum consisted of 1.33 cc. of an eighteen-hour second-generation culture in 1 per cent glucose broth. A massive inoculum was employed to complete the experiment within the fourteen hours. The results are found in table 8.

Reference to figure 7 reveals an interesting point, namely, that the cultures of initial P_H 7.0, 7.5, 8.1 reached practically the same level of hydrogen-ion concentration after eight hours

incubation. To attain this result the cultures of necessity must have produced acid at varying rates. That this was true is brought out by the curves which show a tendency toward convergence after the second hour. From the data in table 8 it

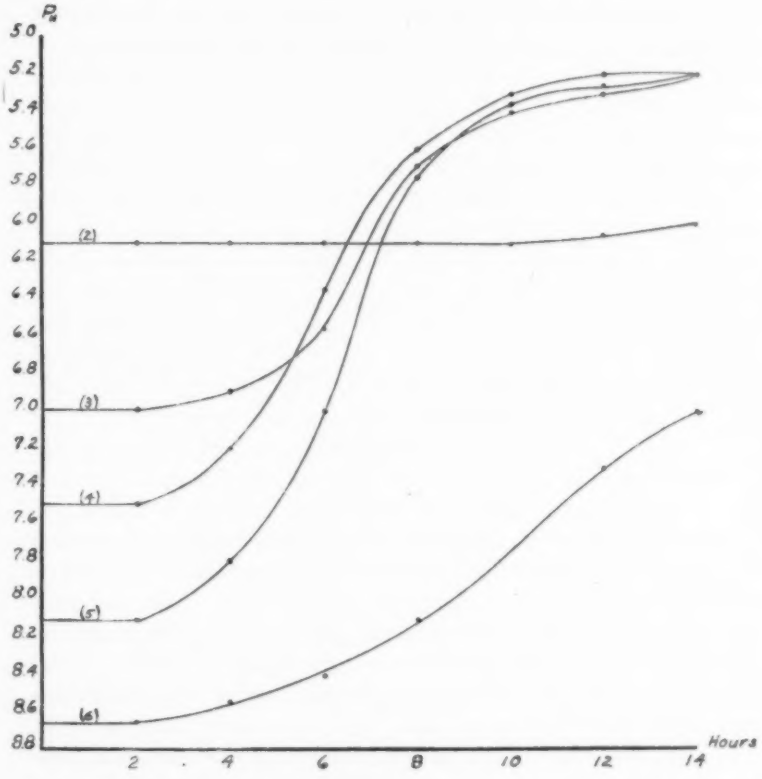

FIG. 7. EXPERIMENT X. THE INFLUENCE OF INITIAL P_H OF BROTH UPON THE RATE OF ACIDITY FORMATION

appears that culture (4) exhibited the shortest lag (two hours) though culture (5) showed the most rapid rate of acid formation during the maximum period, namely, a lowering of 0.625 P_H against a lowering of 0.425 P_H in the cases of (4) and (3). Culture

(6) began its acid formation after two hours at a slow, rather constant rate but at the close of the experiment had only reached a P_H of 7.0. After thirty hours its P_H was 6.0. It was not known whether this culture ever reached the final characteristic hydrogen-ion concentration. Culture (1) showed no growth while (2) was found to grow very poorly, the P_H after thirty hours being at the same level as at the fourteen-hour period.

TABLE 9

Summary

EXPERIMENT	AGE OF PLEURAL FLUID	DURATION STATIONARY PERIOD	DURATION LAG PERIOD	ONSET MAXIMUM PERIOD	P_H (INITIAL)	P_H CHANGE MAXIMUM PERIOD	DURATION MAXIMUM PERIOD	P_H LOWERING	
								Total	Per hour
Medium: 1 per cent glucose broth									
	days	*hours*	*hours*				*hours*		
VI	18	4	4	8	7.10	6.45–5.70	4	1.35	0.34
VII	19	Under2	6	6	7.25	6.70–5.85	2	0.85	0.425
VIII	18	3	5	8	7.20	6.60–5.70	2	0.90	0.45
IX	24	2	6	8	7.65	6.80–5.90	2	0.90	0.45
Medium: 1 per cent glucose, 5 per cent horse serum broth									
VIII	18	2	4	6	7.25	6.70–5.70	2	1.00	0.50
IX	24	2	2	4	7.65	7.20–5.90	2	1.30	0.65

From the foregoing data the following conclusions regarding the rate of acid formation in cultures of *Streptococcus hemolyticus* may be drawn:

1. The curves of acid formation with time may be separated into five characteristic periods: (1) Stationary period, (2) lag period, (3) maximum period, (4) period of negative acceleration, (5) maximum stationary period.

2. It is possible to reduce the duration of the stationary and lag periods to a minimum through increasing the quantity of inoculum. Whether this holds beyond a certain point is not known.

3. The age of the culture that is serving as a source of inoculum may exert a decided effect upon the duration of the stationary and lag periods in the sub-culture. If the inoculum be taken from a culture during its maximum period, lag is reduced to a

minimum in the sub-culture and growth and acid production at a maximum rate are initiated early. This point is of considerable importance, though seemingly it has been overlooked by many workers.

4. The presence of 5 per cent horse serum reduces lag by from two to four hours. This is correlated with an earlier period of multiplication of organisms at a maximum rate. Two possible explanations of this phenomenon present themselves: (1) Nutritive materials in some easily available form may be furnished by the serum or, (2) growth-accessory substances (vitamines) may be present in the enriching fluid. The second possibility would be in accord with Kligler's finding (1919) that the presence of tissue extracts shortened lag in the growth of *Streptococcus hemolyticus* and other organisms. Ordinarily these accessory substances are furnished by disintegrating cells which accounts for the fact that massive inocula give better cultures than light inocula.

5. Entire absence of lag in acid formation has never been noted. One case has been reported above in which a two-hour lag was apparent in glucose-serum broth.

6. In glucose broth the maximum period is initiated between the sixth and eighth hour and is usually maintained for two hours after which the period of negative acceleration sets in. The P_H decrease per hour in this medium is 0.42 (average of four experiments). In glucose-serum broth the maximum periods sets in two to four hours earlier and proceeds for two hours. The P_H decrease per hour during this period is 0.50.

Recent work in this laboratory by Dr. Marjorie W. Cook has demonstrated that hemotoxin production by the "H" strain of *Streptococcus . hemolyticus* occurs nearly always between the sixth and eighth hours. It is a fact of interest that this property appears during the interval which is most frequently associated with maximum acid formation.

7. In glucose broth of initial P_H ranging from 7.10 to 7.65 the maximum period sets in when the P_H of the culture has been brought to 6.45–6.80. The relation of this level of acidity to the optimum P_H of the enzymes associated with acid production might be suggested as a possible explanation of this phenomenon.

8. The initial P_H of broth exerts an effect upon the rate of acid formation. A medium of P_H 7.5 was found to show a minimum of lag, while the most rapid acid formation occurred in broth of P_H 8.1. The optimum P_H of broth for growth and acid production of the "H" strain of *Streptococcus hemolyticus* apparently lies between these two points, P_H 7.5–8.1. Other observers have fixed the optimum P_H of the streptococcus at 7.8.

IV. THE INFLUENCE OF THE INITIAL P_H OF BROTH UPON GROWTH AND ACID FORMATION OF STREPTOCOCCUS HEMOLYTICUS

Before the elaboration of accurate methods for determining the true reaction of a medium much attention was given to the study of the influence of acidity and alkalinity upon the physiological activities of organisms. Unfortunately much of the data obtained in these earlier investigations is of little value owing to the fact that determinations of titratable acidity rather than of true acidity were carried out. The fallacy of titrating media by the older method has been established by Clark (1915a) beyond question and if we are to accept the classic works of Sörensen and Michaelis, as supplemented by a constantly increasing mass of data by other investigators, it must be supposed that the hydrogen-ion concentration rather than the titratable acidity of the environmental medium is the determining factor in regulating the metabolic activities of bacteria and related organisms.

Though it is true that media adjusted by the old titration method may vary considerably in their hydrogen-ion concentrations yet it has been possible in the past to cultivate bacteria with a considerable degree of success. No doubt this has been due rather to the fact that many bacteria are able to develop within a fairly wide range of reaction than to the accuracy of adjustment of the media. The effect of variations in initial P_H would be demonstrable rather in altered rates of growth and fermentation. In the case of some of the more delicate pathogenic bacteria, small variations in reaction may induce very decided effects and it is here particularly that the true reaction

must be carefully controlled. One example may serve to illustrate this point: H. M. Jones (1920) working with the various types of pneumococci found that in a medium of P_H 7.0 no strain was able to develop greater acidity than P_H 5.6, whereas if the initial reaction was P_H 7.6 all strains gave a final hydrogen-ion concentration ranging from 5.0 to 5.4. If the final P_H produced by certain organisms is to serve a useful purpose in differential procedures, the level of the initial hydrogen-ion concentration of the medium must obviously be controlled so as to permit the optimum development of the organism in question, in order that it may carry its fermentation to a maximum.

That there are levels of hydrogen-ion concentration which have the effect of limiting the activities of certain organisms was perhaps first recognized by Lazarus (1908) in 1908, who roughly adjusted her media to various hydrogen-ion concentrations with litmus, phenolphthalein, and methyl orange after which the reactions limiting growth were studied. The influence of reaction was considered a modification of the conditions of assimilation in that it exerted a definite effect upon the state of dissociation of the materials which the organism in question could take up or could alter.

With the recognition by investigators of the growing importance of the relationships of hydrogen-ion concentration to biological process in general, have come attempts to determine the limits of reaction within which bacteria may develop. The most complete single effort to establish such minimum, maximum, and optimum limits of P_H for a number of pathogenic organisms seems to have been that of Fennel and Fisher (1919). In the course of the present investigation it has been possible to collect from a number of sources data bearing on this point and in recognition of the value of a compilation such as this to workers in the field of bacteriology this information has been appended to the present section of the paper.

Experiment XI. The relation of initial hydrogen-ion concentration of broth to the growth of Streptococcus hemolyticus

Portions of infusion broth were adjusted to values ranging from P_H 5.0 to 9.0 and after the addition of proper amounts of glucose and horse serum, were incubated for twenty-four hours to insure sterility. Each tube contained 5 cc. of medium. The following series were used: (1) Plain broth, (2) 1 per cent glucose broth, (3) 1 per cent glucose-5 per cent horse serum broth. The inoculum consisted of 0.2 cc. of an eighteen-hour culture in 1 per cent glucose broth. Duplicate uninoculated tubes were carried as controls. The results are found in table 10.

The following summary will perhaps serve better to express the outstanding points of this experiment:

	PLAIN BROTH	1 PER CENT GLUCOSE-BROTH	1 PER CENT GLUCOSE, 5 PER CENT SERUM-BROTH
Minimum P_H permitting growth	6.35	6.35	5.70
Maximum P_H permitting growth	8.50+	8.50+	9.25+
P_H limits within which luxuriant growth occurs	6.60 8.50	6.35 8.50	5.90 9.25

Whereas the limits of reaction which permit growth appear to be the same in plain and in 1 per cent glucose broth, the presence of horse serum in addition to the glucose enables the organisms to tolerate greater degrees of acidity and alkalinity. Hence it is to be emphasized that in expressing the levels of hydrogen-ion concentration which limit the growth of organisms the exact composition of the experimental media must be mentioned. It has been noted previously that horse serum exerts a strong stimulatory effect upon the growth and fermentative activities of the streptococcus. Here we find additional evidence of such an action in an increased tolerance of the organisms for acidity and alkalinity, manifested by growth throughout a wider range of hydrogen-ion concentration.

From the results of experiment VIII it must be concluded that the optimum P_H, based upon the rate of acid formation in 1

per cent glucose broth, lies between P_H 7.5 and 8.1. If the mean of these two exponents be taken, the value, P_H 7.8, represents the optimum hydrogen-ion concentration for growth and acid production. This corresponds to the optimum found by Fennel and Fisher (1919) for *Streptococcus hemolyticus*. It is interesting to note that this point corresponds exactly to the optimum established for the pneumococcus (see chart) and other pathogenic cocci, and that it is only slightly different from the P_H of human blood.

TABLE 10

Experiment XI

NUMBER	PLAIN BROTH P_H			1 PER CENT GLUCOSE BROTH P_H			1 PER CENT GLUCOSE, 5 PER CENT HORSE SERUM BROTH P_H		
	Initial	48 hours	Control	Initial	48 hours	Control	Initial	48 hours	Control
1	5.00	−	5.0	5.0	−	5.0	5.0		5.0
2	5.30	−	5.4	5.3	−	5.4	5.5	5.4±	5.7
3	5.50	−	5.55	5.5	−	5.55	5.7	5.1+	5.7
4	5.70	−	5.60	5.7	−	5.6	5.9	5.05+++	5.9
5	6.05	−	6.00	6.05	5.95±	6.0	6.3	5.00+++	6.3
6	6.35	5.5++	6.35	6.35	5.20++	6.35	6.4	5.00+++	6.4
7	6.60	6.0+++	6.70	6.60	5.15+++	6.70	6.80	5.00+++	6.80
8	7.00	6.15+++	6.95	7.00	5.25+++	6.95	7.00	5.00+++	7.05
9	7.15	6.40+++	−	7.15	5.20+++	−	7.20	5.00+++	7.20
10	7.45	6.60+++	7.30	7.45	5.1+++	7.30	7.50	5.00+++	7.40
11	7.85	6.80+++	7.50	7.85	5.25+++	7.50	7.70	4.90+++	7.65
12	8.10	7.00+++	−	8.10	5.20+++	−	8.10	5.00+++	8.10
13	8.35	6.90+++	8.25	8.35	5.20+++	8.25	8.30	5.00+++	8.30
14	8.70	8.10++	8.50	8.70	5.20+++	8.50	8.7	5.10+++	8.50
15	9.40	−	8.95	9.40	−	8.95	9.25	5.20++	8.90

− No growth; ± growth doubtful; + fair growth; ++ good growth; +++ excellent growth.

Wolf and Harris (1917a) working with *Clostridium welchii* and *C. sporogenes* have found that the final hydrogen-ion concentration produced by these organisms in media adjusted to different levels is by no means a constant. By constructing curves to show what they term "reaction resultants" an orderly relationship between the point of initial and final P_H was noted. Moreover, in media adjusted within the acid range the character of the "reaction resultant" curve was dependent upon the type of

acid employed in fixing the initial reaction of the medium. Further doubt has been thrown upon the "physiological constant" theory by the work of Wyeth (1918) on *Bact. coli*. By constructing "reaction resultants" such as those suggested by Wolf and Harris (1917a) he was able to show a definite relationship between the initial and final P_H levels. The type of acid employed in adjusting the medium was also found to bear a definite relationship to the final P_H produced by the organisms. From the foregoing results these investigators concluded that no method of clinical differentiation based upon the production of a characteristic level of hydrogen-ion concentration may safely be applied, unless such factors as the initial P_H of the culture medium as well as its composition be very carefully controlled in every test.

Wolf and Harris (ibid.) have directed attention to the fact that fermentations characterized by a slowly decreasing production of acid in the period of depressed acceleration give rise to a final P_H which is a constant regardless of the initial reaction, provided the activities of the organism cease as soon as a definite level of P_H is attained. Expressed differently, the "reaction resultant" appears as a straight line parallel to the abscissa. Seemingly this condition prevails in streptococcus fermentations as table 10 reveals a marked constancy in the levels of final P_H produced in glucose and in glucose-serum media. So far as the initial reaction is concerned it must be concluded that this factor is without influence upon the production of a characteristic hydrogen-ion concentration but that levels of initial P_H which allow growth to occur satisfactorily will also conduce to the attainment of the P_H level established as a "physiological constant" of *Streptococcus hemolyticus*. That the composition of the medium may exert an effect upon the final P_H however, is illustrated in the values obtained with the glucose-serum series (table 10). Here there is a tendency toward the production of slightly higher points of hydrogen-ion concentration, that is, lower P_H levels.

Limits of hydrogen-ion concentration which permit growth of organisms

ORGANISM	REFERENCE	MEDIUM	MINIMUM	MAXIMUM	OPTIMUM
Pneumococcus	Dernby and Avery (1918)	Infusion broth	7.0	8.3	7.8
	Fennel and Fisher (1919)	Infusion broth	7.2	8.2	7.8
	Avery and Cullen (1919b)	Infusion broth	7.0	8.3	7.8
Streptococcus hemolyticus	Fennel and Fisher (1919)	Infusion broth	4.5	8.0	7.6–7.8
	Foster	Infusion broth (1 per cent glucose)	6.35	8.5+	7.8
		Infusion broth (1 per cent glucose, 5 per cent cent horse serum)	5.7	9.25+	
		Infusion broth	6.35	8.5+	
Streptococcus viridans	Grace and Highberger (1920a)	Ascites broth	6.40	8.00	6.8
	Fennel and Fisher (1919)		4.50	8.00	7.6–7.8
Streptococcus erysipelatis	Itano (1916b)				2.24×10^{-8}
Meningococcus	Fennel and Fisher (1919)	Glucose-agar	7.40	7.80	7.6
	Gates	Serum-glucose broth	6.10	7.80	7.4
Gonococcus	Cole and Lloyd (1917)	"Tryptamine B. E."	6.50	9.10	7.6
	Fennel and Fisher (1919)	Starch-agar (Vedder)	7.0	8.00	7.6
Bact. coli	Michaelis and Marcora (1912)	Lactose broth	5.0		
	Shohl and Janney (1917)	Urine	4.6–5.0	9.2–9.6	6.0–7.0
	Wyeth (1918)	Infusion broth	4.30 (HCl)		
	Wyeth (1918)	Infusion broth	4.52 (lactic)		
	Wyeth (1918)	Infusion broth	4.77 (acetic)		

Limits of hydrogen-ion concentration which permit growth of organisms—continued

ORGANISM	REFERENCE	MEDIUM	MINIMUM	MAXIMUM	OPTIMUM
Bact. typhosum	Fennel and Fisher (1919)	Nutrient agar	4.00	9.60	6.2–7.2
	Schoenholz and Meyer (1919)		5.00	8.60	6.8–7.0
Bact. paratyphosum (A)	Fennel and Fisher (1919)	Nutrient agar	4.00	9.60	6.2–7.2
Bact. paratyphosum (B)	Fennel and Fisher (1919)	Nutrient agar	4.00	9.60	6.2–7.2
Bact. dysenteriae (Flexner)	Fennel and Fisher (1919)	Nutrient agar	4.80	9.60	6.2–8.4
Bact. dysenteriae (Shiga)	Fennel and Fisher (1919)	Nutrient agar	4.80	9.60	6.2–8.4
C. welchii	Wolf and Harris (1917a)	Glucose-peptone (2 per cent) water	4.8		
C. metchnikoff	Wolf and Harris (1917a)	Glucose-peptone (2 per cent) water	4.94		
Hemophilus influenzae	Fennel and Fisher (1917a)	Chocolate medium			7.8–8.0
Corynebact. diphtheriae	Bunker (1916–17)		6.30	8.20	6.5–7.5
V. cholerae	Fennel and Fisher (1919)	Extract agar or broth	5.60	9.60	6.2–8.0
B. melitensis	Fennel and Fisher (1919)	Nutrient agar	6.30	8.40	6.6–8.2

Reaction of differential media

OBSERVER	MEDIUM	P_H (MINIMUM)	P_H (MAXIMUM)	P_H (OPTIMUM)
Fennel and Fisher (1919)	Endo			7.8–8.0
Kligler (1918)				7.8–8.0
Fennel and Fisher (1919)	Brilliant green	6.40	7.20	6.8–7.0
Kligler (1918)				7.0–7.2
Meyer and Stickel (1918)				6.4–7.0
Fennel and Fisher (1919)	Russel's double sugar	7.0	7.8	7.4–7.6
Kligler (1918)				7.4

V. THE RELATION OF HYDROGEN-ION CONCENTRATION TO INHIBITION AND DEATH OF STREPTOCOCCUS HEMOLYTICUS

It has long been noted that the growth of a microörganism beyond a certain point exhibits symptoms of inhibition, manifest first in a decreasing growth rate, second by complete cessation of growth, third by a definite decrease in numbers, and finally by death, at which point the culture becomes entirely sterile. Inhibition, representing as it does an almost universal bacteriological phenomenon, ensues from the toxic action of the products of its own metabolism upon the organism itself. Through the continuous accumulation of these waste products in the encompassing medium and through the inability of the organism to escape their contact inhibition becomes more and more pronounced and eventually death supervenes. If the metabolic products are largely of acid nature these substances will exert a harmful effect and if in greater concentrations, a fatal influence. This fact has been well illustrated in the curves of acid formation previously discussed.

Recognizing this principle, Kitasato (1888) in 1888 added various acids to neutral media and then determined the minimum dose required to kill *Bact. typhosum* and *V. cholerae*, and the maximum dose which would still permit their growth. As the results of these experiments were expressed only in terms of percentage concentration they have for us now only historical interest.

Paul and Krönig (1896, 1897) in 1896 pointed out that the toxicity of metallic salts for anthrax spores and for cells of *Staphylococcus aureus* is dependent chiefly upon the effect of the cation but that the anions and undissociated molecules as well may exert a certain influence. Strong acids were found to act in accordance with their concentration of hydrogen ions and to depend to a small extent upon the specific action of the particular anion or undissociated molecules. Winslow and Lockridge (1906) in an extensive study of the toxic effects of certain acids upon colon and typhoid bacilli found that strong acids such as HCl and H_2SO_4 proved fatal in concentrations at which they were highly ionized, whereas weak acids such as acetic and benzoic, proved fatal at concentrations where they were but slightly ionized. In the latter the effect appeared to be due rather to the whole molecules than to the actual concentration of hydrogen-ions.

Paul, Birstein, and Reuss (1910a) attributed a considerable toxic influence to the acid anion present as well as to the undissociated molecules. The toxic action of hydrogen-ions upon the cell appeared to be catalyzed by anions. This was found to be especially true of the weak organic acids. This finding has been supportéd by Norton and Hsu (1916) who added that the undissociated molecules act as negative catalyzers of the action of the hydrogen-ions. Addition of a salt having the same anion as the acid in question was found to decrease the disinfecting power through depression of the hydrogen-ion concentration (common ion effect), though the retarding influence appeared to be greater than would be expected from the decreased hydrogen-ion concentration alone. Salts not appreciably affecting the ionization of the acid brought about an increase in disinfecting power. These conclusions are not in accord with other results reported by Paul, Birstein, and Reuss (1910b) These observers showed that salts which exercised no disinfecting power in themselves were capable of increasing the toxicity of inorganic acids having the same or different anions.

A direct relationship between the degree of ionization of acids and their toxicity for yeast cells was reported by Bial (1902)

who accordingly divided the acids used into three classes based upon their ionization constants and similarity in toxicity. Surprising differences in the toxicity of various acids for molds were found by J. F. Clark (1899) in 1899. The degree of dissociation seemingly stood in no relation to the toxicity and this observer was forced to the conclusion that the inhibitory property, for molds at least, resided largely in the undissociated molecules.

The approximate concentrations of a number of common inorganic and organic acids required to inhibit growth of *Streptococcus pyogenes* have been determined by Taylor (1917) in the course of studies on the disinfection of war wounds. Considerable variation in potency was apparent with the organic acids investigated though apparently no account was taken of their degrees of ionization.

Wolf and Harris (1917a) in their study of the effect of acids upon the fermentations of *Clostridium welchii* and *C. sporogenes* point out that the influence is two-fold; first, that exerted by the hydrogen-ions, and second, that due to the anions and undissociated molecules. Lactic acid was found to have about the same toxicity as hydrochloric, whereas acetic, succinic, and butyric inhibited growth at lower hydrogen-ion concentrations (higher P_H). Wyeth (1918) reported similar results with *Bact. coli*. He points out that if the actual mass of acid be considered hydrochloric was more inhibitory than lactic or acetic acids but that the lethal points of such organic acids, in terms of hydrogen-ion concentrations, were lower than that of hydrochloric. In equivalent quantities the highly ionized acids proved more effective in inhibiting growth.

Lord (1919), has obtained data which lead him to believe that acidity is the principal inhibitory factor in glucose broth cultures of pneumococcus, though H. M. Jones (1920) very recently has succeeded in demonstrating that in the presence of body fluid such as blood serum or ascitic fluid the tolerance of this organism for hydrogen ions is considerably increased. This same phenomenon had been noted previous to the appearance of Jones' article during the course of the present investigation upon *Streptococcus hemolyticus* and the facts have proved so

interesting that they will be presented in this section of the paper.

In numerous experiments it has been observed that a glucose broth culture of *Streptococcus hemolyticus*, after reaching a stationary level of hydrogen-ion concentration during the first twenty-four hours, remains viable for a period varying from two to five days. Subcultures made on each succeeding day during this period of death show stationary and lag periods of increasing duration. To gain some idea of the factors contributing to this inhibition the following experiments were carried out:

Experiment XII. The growth and acid production of Streptococcus hemolyticus in neutralized filtrates

A transplant of the usual quantity of an eighteen-hour culture was made into 1 per cent glucose broth, P_H 7.5, and the material incubated until sterile (five days). The culture was then filtered through a sterile Berkefeld candle, after which the filtrate was brought back to the original reaction with sterile NaOH and re-inoculated with a fresh, actively growing culture. This procedure was repeated until no further growth resulted upon inoculation. The results are found in table 11.

TABLE 11
Experiment XII

FILTRATE	FINAL P_H	GROWTH	BROUGHT TO P_H
1	5.10	+++	7.60
2	5.00	+++	7.50
3	5.30	+++	7.80
4		−	

From the data shown in table 11, it would appear that acidity is the chief factor causing inhibition and death of the streptococcus in glucose broth cultures. The inhibition which finally appears may be due to two factors; first, to an exhaustion of nutrient materials in the medium, and second to the accumulation of toxic products other than acid which check metabolism and growth.

Chesney (1916) in a rather extensive investigation of the latent period of bacteria noted variations in the toxicity of filtrates, taken at intervals following the maximum period from plain broth cultures. Inhibition appeared strongest at the time when the culture had attained the summit of its growth and became progressively less as the period of incubation increased. At the point where the culture became sterile a minimum of inhibition was shown. Filtrates taken early in the maximum period of growth showed no inhibitory property while those taken near the end of the same period proved to be somewhat toxic. According to Chesney the inhibitory substances represent waste products of the bacterial cells or unused portions of food molecules, and the alteration of the cells occasioned by their exposure to these toxic materials is concerned with that structure or function which is essential to metabolism and hence to growth. It must be emphasized that in Chesney's experiments plain broth cultures were studied and that consequently the factor of acidity was absent. In fact no determinations of hydrogen-ion concentration were carried out.

It is a well recognized fact that plain broth cultures of the streptococcus remain viable throughout much longer periods than do glucose-broth cultures of the organism. This would tend to substantiate the conclusion drawn from experiment XII that acidity is the chief single factor causing inhibition and death of the streptococcus. Natvig (1909) in an investigation of acid production by the streptococcus arrived at the same conclusion.

Refrigeration of streptococcus cultures is known to be one of the best means of maintaining the viability of the organisms and it has been observed in this laboratory that such a procedure is especially useful in preserving the pleuritic exudates employed as a source of culture material in the present investigation. It would be expected that the decrease in temperature occasioned in transferring a culture from the incubator to the ice chest would reduce the rates of metabolism and growth to a low level. As a consequence the toxic products of bacterial metabolism would increase in the medium at a much slower rate than if the culture were incubated. Obviously this condition would tend to preserve the viability of a culture for long periods.

Experiment XIII. The inhibitory action of acids upon a culture of Streptococcus hemolyticus

One per cent glucose broth was inoculated as usual with an eighteen-hour actively growing culture and permitted to incubate for eighteen hours. At the end of this interval a portion was filtered with sterile precautions through a Berkefeld candle and another portion was centrifugalized. P_H determinations were then made upon the supernatant and the filtrate. Portions of beef infusion broth containing 0.5 per cent KH_2PO_4 (to aid in maintaining the reaction) and 0.5 per cent glucose were next adjusted to the P_H levels of the cultures, using the acids indicated in table 12. The supernatant fluid, Berkefeld filtrate, and tubes containing the broth adjusted with acids were inoculated with equal amounts of an eighteen-hour culture in 1 per cent glucose broth. Tests of viability were carried out by streaking one loopful of material on the surface of blood-agar plates at hourly intervals. As will be seen by reference to table 12 some of the tubes contained 5 per cent horse serum.

In the cases of (2) and (4) the addition of 5 per cent horse serum caused a change in P_H toward the alkaline side and consequently the results in these tubes are not comparable with the others. The rather close agreement in toxicity between lactic (1) and acetic (2) acids at the same P_H is of interest. The mixture of the two acids in molecular proportions killed in twelve hours, but inasmuch as the P_H of this tube was 5.15 as against 5.25 in (1) and (3) the result cannot be considered as evidence of increased toxicity. By comparing (6) with (1), (3), and (5) the protective action of horse serum is strikingly illustrated. Tube (6) contained viable cells after fifty-four hours contact with an acidity of 5.20; in other words, the streptococci were able to tolerate the same degree of acidity for a period nearly four times longer when in contact with 5 per cent horse-serum. Close agreement between the toxicities of the supernatant and filtrate are apparent ((7) and (8)) though neither proved as toxic as lactic or acetic acids of the same hydrogen-ion concentration.

TABLE 12

Experiment XIII

VIABILITY AFTER HOURS	ACID								
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	Lactic, P_H 5.25	Lactic, 5 per cent horse serum, P_H 5.40	Acetic, P_H 5.25	Acetic, 5 per cent horse serum, P_H 5.40	Lactic, acetic, P_H 5.15	Lactic and acetic, 5 per cent horse serum, P_H 5.20	Supernatant, P_H 5.20	Filtrate, P_H 5.30	Lactic, P_H 4.95
6	+++	+++	+++	+++	++	+++	+++	+++	+
7	+++	+++	+++	+++	++	+++	+++	+++	±
8	+++	+++	+++	+++	60++	+++	+++	+++	−
9	+++	+++	+++	+++	10+	+++	+++	+++	−
10	++	+++	+++	+++	6+	+++	+++	+++	−
11	+	+++	+++	+++	1−	+++	+++	+++	−
12	+	+++	++	+++	−	+++	+++	+++	−
13	±	+++	35++	+++	−	+++	+++	+++	−
14	−	+++	21++	+++	−	+++	+++	+++	−
15	−	+++	6+	+++	−	+++	+++	+++	−
16	−	+++	−	+++	−	+++	+++	+++	−
17	−	++	−	+++	−	+++	++	++	−
19	−	60++	−	++	−	+++	14+	13+	−
21	−	10+	−	60++	−	++	5+	12+	−
32	−	5+	−	24+	−	35+	−	−	−
36	−	4+	−	4+	−	23+	−	−	−
54	−	−	−	2±	−	7+	−	−	−

Numbers represent colonies developing from one loopful of culture.

+++ Profuse growth; ++ good growth; + growth sparse (less than 50 colonies); ± growth very doubtful (one or two colonies); − no growth after twenty-four hours incubation.

SUMMARY

1. Streptococcus hemolyticus is able to ferment the common hexoses and disaccharides but not the polysaccharides. The final hydrogen-ion concentration produced in broth containing different sugars varies between the limits P_H 4.85–5.40. The lowest P_H is registered in broth containing glucose; the highest P_H in broth containing lactose. The characteristic final P_H is seldom reached in the first generation but is usually attained by

the second generation culture. Subsequent transplants do not show lower levels of P_H.

2. Plain broth cultures of *Streptococcus hemolyticus* show a decrease in P_H which is practically the same as that exhibited by cultures of the organism in sugar-free broth. This is believed to be due to a selective action upon that portion of the peptone molecule which Pick has shown reacts typically like a carbohydrate.

3. *Streptococcus hemolyticus* is able to produce its characteristic final P_H in neutral broth containing 0.2 per cent glucose. Concentrations of glucose up to 1 per cent have no further effect upon the level of the final P_H.

4. The final hydrogen-ion concentration of the streptococcus is not influenced by the presence of K_2HPO_4 in concentrations up to 1 per cent providing sufficient glucose is present.

5. Titration curves show that horse serum in broth exerts a slight but distinct buffer effect.

6. The curves of acid formation with time may be separated into five characteristic periods; (1) stationary period, (2) lag period, (3) maximum period, (4) period of negative acceleration, and (5) maximum stationary period.

7. Through an increase in the amount of inoculum or by employing a parent culture of suitable age as a source of inoculum it is possible to reduce the stationary and lag periods to a minimum.

8. The presence of 5 per cent horse serum in glucose broth reduces lag in acid formation by two to four hours. This may be due to, (1) the presence of growth-accessory substances, or (2) the presence of easily available nutritive materials.

9. In glucose broth the maximum period of acid formation is initiated usually between the sixth and the eighth hours and is maintained for two hours. Maximum production of hemotoxin has been found to occur between the sixth and the eighth hours.

10. The most rapid formation of acid takes place in broth adjusted to a P_H of 8.1, while a minimum of lag is shown in broth of P_H 7.6. The optimum P_H for acid formation is believed to lie between these two levels, or at 7.8.

11. The limits of hydrogen-ion concentration which support growth of *Streptococcus hemolyticus* are as follows:

P_H	PLAIN BROTH	1 PER CENT GLUCOSE BROTH	1 PER CENT GLUCOSE, 5 PER CENT SERUM BROTH
Minimum permitting growth	6.35	6.35	5.70
Maximum permitting growth	8.50+	8.50+	9.25+
Limits permitting luxuriant growth	6.60−	6.35−	5.90−
	8.50	8.50	9.25

12. Acidity is the chief factor contributing to inhibition and death of the streptococcus in glucose broth cultures. This is evidenced by the fact that growth proceeds luxuriantly in filtrates from active cultures the acidity of which has been neutralized by a base.

13. At a P_H of 5.25 lactic and acetic acids appear to have about equal disinfecting powers for *Streptococcus hemolyticus*. Organisms live for longer periods in filtrates from active cultures than in broth brought to the same P_H with either lactic or acetic acids.

14. A marked increase in tolerance for acid is shown by streptococci in the presence of horse serum. In one test it was found that viability persisted for a period nearly four times as long in serum-glucose broth of P_H 5.20 as was evident in glucose broth adjusted to the same P_H.

THE BIOCHEMISTRY OF STREPTOCOCCUS HEMOLYTICUS

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I. THE ACIDS PRODUCED BY STREPTOCOCCUS HEMOLYTICUS[1]

The cessation of activity in a glucose broth culture of *Streptococcus hemolyticus* comes when a fairly constant point of hydrogen-ion concentration is reached. This change is caused by a fermentation of the medium with a resultant formation of acid substances. The present section of this paper has to do with the chemical nature of these acid products.

A review of the literature reveals the fact that scant attention has been paid to the biochemistry of bacterial fermentations. Emmerling (1896), in 1896, carried out determinations of acids obtained from the putrefaction of certain proteins through the action of *Proteus* and *Staphylococcus pyogenes*. Tissier and Martelly (1902) in a study of the putrefaction of meat found that *Streptococcus pyogenes* could only split natural proteins after the latter had been peptonized. The same organism rapidly attacked glucose forming, chiefly, lactic acid. *Clostridium welchii* and *C. sporogenes* were found to produce acid and alkaline substances simultaneously. Later work by Wolf and Telfer (1917) upon these last-mentioned organisms has shown that a large proportion of the acid formed in their fermentations is volatile in character. The method of Dyer (1916) was employed by these investigators in determining the volatile acids. Forty per cent of the total acid produced by the organisms mentioned proved to be non-volatile. The exact chemical nature of this fraction was not determined.

[1] Miss Bernice Rhodes rendered valuable assistance in carrying out the experimental work described in this section of the paper.

In a study of the acid fermentation of xylose by Fred, Peterson, and Davenport (1919) the main products were found to be acetic acid and lactic acid. The proportion of volatile acid to non-volatile acid proved to be the same throughout the entire ten to twelve days of fermentation; namely, 40 per cent of acetic to 60 per cent of lactic acid. The two acids represented about 90 per cent of the sugar consumed.

Speakman (1920) investigated the biochemistry of acetone and butyl alcohol fermentation of starch and showed that acetic and butyric acids are formed as intermediate products. A reduction of these acids to the corresponding alcohol subsequently sets in.

Methods

Volatile acids. Volatile acids were determined by the steam distillation method of Duclaux (1900) as modified by Dyer (1916). This procedure is one in which the acid solution is distilled with steam at a constant volume. The distillate is collected in 10 cc. fractions until 100 cc. have passed over, after which the remainder is taken off in 100 cc. portions. These fractions are then titrated with N/50 alkali, using phenolphthalein as indicator, and the percentage of acid is calculated. The amount of acid in a given fraction, the "distilling constant" for the fraction, is plotted against the corresponding portion of distillate on logarithmic coordinate paper. Pure acids are graphically represented as straight lines and arrange themselves consecutively from the lower to the higher members of the series. With a mixture of two volatile acids the first part of the curve occupies a position intermediate to the lines representing the distilling rates of the higher and lower boiling acids. As the higher boiling acid is removed, the curve gradually becomes parallel with the line representing the lower boiling acid.

It follows that the distillate from an unknown acid mixture may be approximately determined by plotting a curve from the distilling constants and comparing this with the curves established for known acids.

The apparatus employed differed from that of Dyer (1916) in that the heating of the flasks was done with gas instead of electricity. By thoroughly insulating the flasks and connecting tubes with asbestos it was possible to maintain a constant volume throughout a long distillation.

To test the accuracy of the method several preliminary determinations of pure acids were first carried out. The results obtained for formic, acetic, and caproic acids are to be found in

TABLE 1

Distilling constants (pure acids)

FRACTION	FORMIC ACID	ACETIC ACID	CAPROIC ACID
cc.			
10	2.0	4.66	24.55
10	3.8	9.45	43.85
10	5.5	14.17	58.83
10	7.5	18.93	70.25
10	9.8	23.57	78.30
10	12.0	28.16	83.90
10	14.0	32.59	88.16
10	16.4	36.91	91.46
10	19.0	41.19	93.90
10	21.2	45.24	95.74
100	35.6	71.89	99.22
100	52.4	89.60	99.95
100	63.5	99.49	

table 1. Graphical representation of the distilling rates has been made on logarithmic coordinate paper (see fig. 1). The curves were found to fall between those of Dyer and those of Wolf and Telfer. Attempts to use the color tests suggested by Dyer did not meet with success and accordingly they were abandoned.

Lactic acid. It was presumed that the non-volatile portion of the cultures consisted mainly of lactic acid; therefore the following quantitative method suggested by Fred, Peterson, and Davenport (1919) was employed for its determinations:

The residue from the distillation flask was carefully evaporated on a hot plate to a volume of about 40 cc. This was placed in a Soxhlet extractor and extracted with ether for fifty to sixty hours

to remove the lactic acid. About 30 cc. of water were added to the ether extract and the ether removed by distillation. The water extract remaining was then titrated with N/10 $Ba(OH)_2$ adding a 5 cc. excess of the base. The material was boiled for fifteen minutes to convert all of the lactic acid to the barium salt, after which the excess of $Ba(OH)_2$ was removed by neutralization with H_2SO_4. This mixture was allowed to stand for several hours on a steam bath, filtered, and the filtrate and

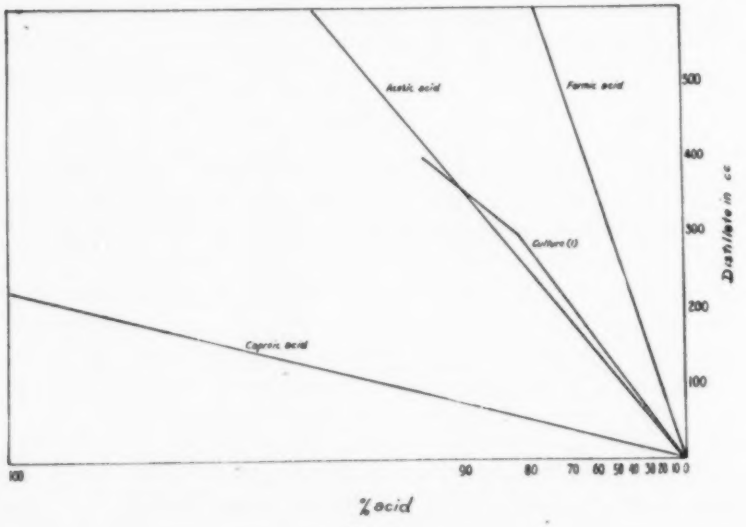

FIG. 1

washings evaporated to dryness. After taking up the residue with water the material was again filtered to remove traces of organic matter or carbonates. The filtrate was then made up to a definite volume (50 or 100 cc.) and a 10 or 20 cc. aliquot taken for analysis. A test for succinic acid was made at this point by adding to the aliquot sufficient 95 per cent alcohol to bring the volume to 100 cc. Succinic acid, if present, gives a precipitate, and the material must be filtered. The filtrate was again evaporated to dryness and the residue taken up in 60 per

cent alcohol. This alcoholic solution was carefully evaporated to dryness in a tared dish and dried at 130°C. to a constant weight. An excess of H_2SO_4 was next added and the material converted to $BaSO_4$ by ignition. From the weight of $BaSO_4$ obtained, the corresponding weight of lactic acid was readily calculated. In case lactic is the only acid present the theoretical yield of $BaSO_4$ may be estimated from the weight of dried barium salt previously found.

Experiment I. The volatile and non-volatile acids produced in streptococcus fermentations

One hundred and fifty cubic centimeters of broth were inoculated with 6 cc. of a first-generation, glucose broth culture of *Streptococcus hemolyticus* and incubated for eighteen to twenty-four hours. Determinations of the volatile and non-volatile acids were made upon 100 cc. of this culture in the manner described. Control determinations were carried out upon a sample of uninoculated broth. The following results were obtained:

Acids	*Broth*
Volatile (as cc. of N/10 acid per 100 cc. broth)	4.42
Lactic acid (as grams per 100 cc. broth)	0.031

The values shown in the table were applied as corrections in the analyses of cultures.

Of the numerous estimations which have been made the results of but three will be presented: Culture (1), 1 per cent glucose broth; culture (2), 1 per cent glucose broth; culture (3), 1 per cent glucose, 5 per cent horse serum broth.

The curves plotted from the "distilling constants" (shown in table 2) proved to be so closely analogous that only one result will be shown. From the position of the line (fig. 1) representing the distilling rate of culture (1) it would appear that acetic acid, chiefly, is elaborated by the streptococcus during its growth in broth media containing either glucose or glucose plus horse serum. A trace of formic acid may also be present. This conclusion is the only one that may be drawn at present even though the

curve is not typical of a mixture of two volatile acids. No attempt has been made to re-fractionate the distillates, consequently the percentage of each acid in the volatile portion is not known. Reference to table 3 indicates that no close agreement exists between the per cents of volatile acid from culture (1) and culture (2) though the same lot of broth was used in each and the fermentation apparently progressed to the same point as the final P_H of the cultures was practically equal. Culture (3) gave a fraction of volatile acid still smaller than was noted in the other cases.

TABLE 2

Experiment I

FRACTION	CULTURE (1)	CULTURE (2)	CULTURE (3)
cc.			
10	4.50	5.32	3.45
10	9.00	9.06	7.13
10	13.81	12.07	10.58
10	18.68	15.71	13.32
10	23.83	18.50	16.01
10	28.08	21.82	18.69
10	32.63	24.61	21 :8
10	35.84	27.62	24.07
10	39.26	30.10	27.05
10	42.14	32.36	30.26
100	61.19	59.40	60.06
100	77.32	77.52	76.56
100	93.45	91.17	88 26
100	99.89	100.90	99.96

The lactic acid estimations in the two glucose broth cultures do not correspond especially well and the value obtained in culture (3) is smaller than would be expected.

Through a lack of time it has been impossible to carry the present phase of the investigation to a logical completion. As a consequence it will be inadvisable to draw other than very general conclusions from the data presented.

It may be concluded from results that are to be presented later that in a 1 per cent glucose broth culture of the streptococcus some 156 mgm. of glucose are utilized in the first twelve to eighteen hours. From the data shown in table 3 it is possible

to calculate the amount of glucose destroyed in the formation of the acids. Assuming for the moment that the volatile fraction consists entirely of acetic acid and the non-volatile fraction of lactic acid, a calculation indicates that the total acidity of this culture accounts for only 50 per cent of the glucose utilized. Fred, Peterson, and Davenport (1919) were able in this way to account for 90 per cent of the sugar utilized in their xylose fermentations. The large discrepancy in the present experiment may possibly be due to two factors, first to experimental error, and second to the fact that another unknown non-volatile acid is present in the fermentation mixtures.

TABLE 3

Experiment I

NUMBER	CULTURE	P_H		VOLATILE ACID (N/10 ACID PER 100 cc.)	LACTIC ACID		VOLATILE ACID	LACTIC ACID
		Initial	Final		Per 100 cc.	N/10 acid per 100 cc.		
				cc.	*grams*	*cc.*	*per cent*	*per cent*
1	Glucose broth	7.4	5.3 (18 hrs.)	1.196	0.061	6.78	15.0	85.0
2	Glucose broth	7.4	5.4 (18 hrs.)	2.98	0.0725	8.05	27.1	72.9
3	Glucose serum broth	7.4	4.9 (24 hrs.)	2.68	0.0534	5.25	33.8	66.2

II. THE METABOLISM OF STREPTOCOCCUS HEMOLYTICUS

Within the past few years evidence has been increasing which indicates that bacterial metabolism and human cellular metabolism have certain fundamental characteristics in common. We are indebted principally to Kendall and his collaborators for our more definite knowledge of the chemical activities of unicellular organisms. Cellular metabolism consists of two distinct phases—(1) the anabolic or structural phase, (2) the katabolic, destructive or "fuel" phase. As in the case of man, bacteria obtain structural material from nitrogenous nutrients while the energy requirements are best satisfied by carbohydrate substances. The analogy may be extended farther to the well known physiological principle that "Carbohydrates spare body nitrogen." In other words, those bacteria which are capable of utilizing both carbo-

hydrate and protein for katabolic purposes will attack the former in preference to the latter (Kendall and Farmer, 1912a, 1912b). This phenomenon, which has been established by Kendall and his associates as a fundamental principle of bacterial metabolism, may be expressed concisely, according to Kendall and Farmer (1912d) in the statement that, "Fermentation takes precedence over putrefaction." These authors define fermentation as, "The action of microorganisms upon carbohydrates, putrefaction as the action of microorganisms upon nitrogenous substances." They state further that "The products of proteolytic activity, which are only formed when bacteria are utilizing protein for fuel are alkaline nitrogenous substances; the products of fermentation, on the contrary, which are formed when bacteria are utilizing carbohydrates for fuel, are non-nitrogenous, acid products."

Inasmuch as nitrogen is the most important structural element entering into the composition of the cell, a quantitative measure of nitrogen degradation must form a very important step in the study of cellular metabolism. In man, nitrogenous waste is excreted from the body mainly as urea, but with bacteria, which are known to excrete nitrogen principally as ammonia, urea, if formed at all, would represent a product of intermediary metabolism. This theory is borne out by the fact that certain bacteria are able actually to utilize urea.

Kendall and his associates (1913) have concluded after many studies upon a variety of organisms that ammonia formation, representing the final step in the degradation of proteins and protein derivatives, is the best available index of proteolysis by bacteria. Ammonia formation is considered by Kendall and Walker (1915) to result from intracellular deaminization of assimilated protein derivatives, incidental to their transformation into energy.

Kendall, Day, and Walker (1913a) have estimated that the amount of protein needed for structural purposes by the bacterial cell is in all probability exceeded by the amount wasted through excretion. The combined structural needs and structural waste are much less than the fuel needs and the fuel waste. Further, the fuel requirements only cease upon the death of the organism,

whereas the structural needs are practically complete when the cell attains its morphological maturity. Consequently the fuel requirement is one of comparatively long duration. According to the same authors, a rapid disintegration of fuel materials occurs in the case of saprophytic bacteria. In other words, such microorganisms must, in general, be considered more active chemically than are pathogenic bacteria.

In the experiments conducted with the streptococcus it has been noted that growth in vitro is always accompanied by elaboration of acid products through the fermentation of materials of carbohydrate nature. No medium has ever been used which does not respond to the fermentative activities of this organism. Kendall, Day, and Walker (1913b) state that when bacteria are metabolizing carbohydrate the nitrogen requirement is minimal, so that in glucose broth cultures of *Streptococcus hemolyticus* we would expect the katabolic or "fuel" phase to predominate over the anabolic or structural phase of metabolism.

Moreover, the presence of horse serum in broth was found to exercise a decided stimulatory effect upon growth rate and acid formation, and also proved effective in permitting growth throughout a wider range of hydrogen-ion concentration. From a consideration of the fundamental features of bacterial metabolism as outlined in the foregoing discussion, it would seem obvious that these phenomena represent a stimulated metabolism of the organisms brought about through some property of the serum. It was suggested previously that structural or growth-accessory substances are perhaps furnished by this material thus permitting the organisms to inaugurate their metabolic activities earlier with consequent reduction of lag. This theory would be in accord with the statement of Kendall, Day, and Walker (1913b) that the structural function always precedes the vegetative or fuel function chronologically, inasmuch as the cell must be formed before it can carry on its appropriate activities.

The following experiments represent an attempt to study the metabolism of the streptococcus in various culture media with an especial effort to determine whether correlation exists between the rates of acid formation and the rates of other metabolic processes in (1) glucose broth, and in (2) glucose-serum broth.

Methods

Ammonia was determined by the Folin air current method (1912) using 2 cc. of culture and collecting the gas in N/50 acid after which the residual acid was determined by back titration with N/50 base. Results are expressed as milligrams per 100 cc. of culture.

Amino acids were determined by the formol titration method of Sörensen previously described (see section I).

Glucose was determined by the method of Bertrand (Hawk, 1918). As the presence of peptone and protein material in the medium rendered the application of the method impossible, the following procedure, devised by Dr. Marjorie W. Cook, was employed to free the cultures from interfering substances:

Twenty cubic centimeters of culture was diluted to 100 cc. with distilled water and precipitated with 10 to 15 cc. of saturated tannic acid solution. After filtering, 5 to 7 grams of lead acetate were added to the filtrate to remove excess of tannic acid and this mixture was filtered. If the filtrate was turbid more lead acetate was added. To the filtrate from this treatment was added 2 to 3 grams of sodium oxalate. This removed the lead as $Pb(C_2O_4)$. The filtrate from this last treatment should be perfectly clear and colorless. It is important throughout the whole procedure to keep the containers and funnels covered thus minimizing evaporation and reducing the error from this source. Two 10 cc. portions of this liquid were now used for determinations of glucose.

Bacterial counts were made by the method of Wright and logarithms of the values so obtained were employed in plotting growth curves. It must be borne in mind that the method of Wright gives only approximate results and that the values represent the total number of organisms rather than the number of viable cells.

Titrations of hemotoxin were made with sterile tubes, pipettes, etc., to maintain the purity of the streptococcus cultures. Rabbit corpuscles which had been washed three times in 0.85 per cent NaCl and made up in a 1 per cent suspension in beef infusion

broth were used in the tests. To 0.5 cc. of the corpuscular suspension were added amounts of culture varying from 0.005 cc. to 0.5 cc. The volume was then made up to a total of 1 cc. with broth, after which the mixture was incubated at 37° for two hours. During the first hour of incubation the tubes were frequently shaken to insure thorough mixing. At the end of the incubation period the degree of hemolysis was observed, and expressed as follows:

100 per cent of corpuscles hemolyzed ++++
90 per cent of corpuscles hemolyzed +++±
75 per cent of corpuscles hemolyzed +++
50 per cent of corpuscles hemolyzed ++
25 per cent of corpuscles hemolyzed +
0 per cent of corpuscles hemolyzed −

Experiment II. The "protein sparing" action of Streptococcus hemolyticus

Bacto beef broth, P_H 7.2, served as the basis of the combinations used in the experiment. The inoculum for each 10 cc. of broth consisted of 0.4 cc. of an eighteen-hour, glucose broth culture. The ammonia determinations were made after seventy hours incubation. The results are incorporated in table 4.

TABLE 4
Experiment II

NUMBER	SERUM	GLUCOSE	FREE NH_3 AS MGM. N PER 100 CC. (CULTURE)	FREE NH_3 AS MGM. N PER 100 CC. (CONTROL)
	per cent	*per cent*		
1	—	—	9.80	9.73
2	5	—	13.02	8.55
3	—	1.0	9.25	7.85
4	—	0.1	10.22	7.00
5	—	0.3	9.95	8.00
6	5	1.0	9.95	8.68

The results show that in the presence of little or no free carbohydrate, as in (2) and (4) of the table, the ammonia output is slightly increased over that found in the presence of 1 per cent glucose. If protein material in the form of horse serum be present the ammonia output is distinctly higher. This is shown

in (2). When the 5 per cent horse serum is in the presence of 1 per cent glucose, however, no increase in NH_3 is manifest ((6) in the table) showing that the sugar shields the protein from attack in this instance.

Experiment III. The protein and carbohydrate metabolism of Streptococcus hemolyticus in broth containing (1) glucose and (2) glucose plus horse-serum[2]

Three hundred cubic centimeter lots of media of the following composition were prepared from beef infusion broth, P_H 7.3: (1) 1 per cent glucose broth; (2) 1 per cent glucose, 5 per cent horse serum broth; (3) same as (2).

(1) and (2) were inoculated with 12 cc. of an eighteen-hour, second-generation culture from pleural fluid no. 198 in 1 per cent glucose-broth. (3) was inoculated with an equal amount of an eighteen-hour, first generation culture of the so-called laboratory strain. This culture differs from the pleural fluid culture in that it has been repeatedly transplanted upon artificial culture media since the original isolation, whereas the latter has been passed many times through rabbits. Both cultures were carried in this experiment in order to determine if repeated transplantation upon artificial culture media had brought about changes in the strain which might appear as an alteration of some phase of its metabolism.

The following determinations were carried out upon samples removed with aseptic precautions at intervals of three hours: (1) Bacterial counts; (2) P_H; (3) glucose; (4) ammonia; (5) amino acids; (6) hemotoxin.

The experiment extended through a period of twelve hours. Table 5 contains the experimental data.

The curves of growth, acid[3] formation and glucose utilization in the three cultures are found in figures 2, 3, and 4. It will be

[2] The writer desires to express his appreciation for the assistance rendered by Dr. Marjorie W. Cook and Miss Bernice Rhodes in carrying out this experiment.

[3] The term acid is used to express true acidity in terms of P_H.

noted that although a rough parallelism between these factors is shown in the three curves of each culture it is most striking in the pleural fluid culture (fig. 3). Here, particularly in the maximum period, may be seen a close relationship between the curves of growth, acid formation, and glucose utilization. A correspondence of the three factors in the maximum period

TABLE 5

Experiment III

TIME	BACTERIAL COUNT		P_H	GLUCOSE		NH_3, MGM. N PER 100 cc.	AMINO ACIDS, MGM. N PER 100 cc.	HEMOLYSIS
	Per cu. mm.	Log		Per 100 cc.	Utilized			
(1) 1 per cent glucose broth (pleural fluid no. 198)								
				grams	*mgm.*			
0	16,400	4.21	7.30	1.09	0	6.01	18.87	0
3	50,700	4.71	7.20	1.172	0	5.73	19.07	0
6	960,000	5.98	6.40	1.007	89	6.57	17.73	purple
9	1,140,000	6.06	5.75	0.955	141	9.93	17.57	+++
12	1,700,000	6.23	5.55	0.935	161	9.79	19.51	0
(2) 1 per cent glucose, 5 per cent horse-serum broth (pleural fluid no. 198)								
0	23,800	4.38	7.35	1.096	0	5.03	19.77	0
3	90,200	4.96	7.20	1.068	28	6.29	18.21	0
6	2,000,000	6.30	5.80	0.941	155	9.93	16.87	++++
9	3,500,000	6.54	5.10	0.877	219	10.49	19.41	++++
12	4,560,000	6.65	4.90	0.847	249	10.07	20.73	0
(3) 1 per cent gucose 5 per cent horse-serum (Laboratory strain)								
0	40,000	4.60	7.35	0.992	0	6.43	19.17	0
3	90,400	4.96	7.20	1.001	0	5.45	20.95	0
6	1,456,000	6.16	5.90	0.862	130	8.67	18.03	++++
9	2,860,000	6.46	4.95	0.722	270	8.25	20.55	++++
12	4,700,000	6.67	4.90	0.728	270	7.69	20.61	0

appears in the other two cultures as well, though it is less striking. In each case it will be observed that a rise in the acidity curve is preceded by a rise in the growth curve. Attention should be directed to the fact that while acid production proceeds most rapidly during the time when the organisms are multiplying at a maximum rate, nevertheless, a considerable lowering of

P_H occurs during the succeeding period when the cells are increasing at a diminishing rate. Stated differently, the curves of growth in each culture depress more sharply from the maximum period than do the curves of glucose utilization and acid formation. Reference to table 5 shows that hemotoxin production commenced in the serum cultures by the sixth hour and persisted

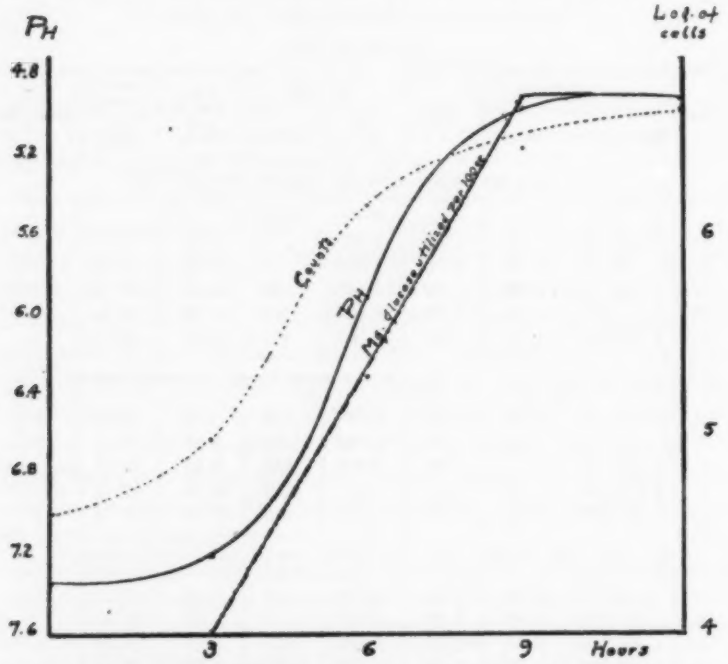

FIG. 2. EXPERIMENT III. CULTURE (1); 1 PER CENT GLUCOSE BROTH

for at least three hours. In the glucose culture however, no definite appearance of hemotoxin was evident until the ninth hour. In each case hemotoxin appears in the period characterized by growth and acid formation at decreasing rates.

Reference to figures 5, 6, and 7 indicates that ammonia production was undergoing a definite increase by the third hour in each of the cultures. This increase in the serum cultures (figs.

6 and 7) was greatest between the third and the sixth hours while in the glucose culture (fig. 5) it was largest between the sixth and the ninth hours. The increase in ammonia output in a general way parallels growth and acid formation during the maximum period in each case. Associated with this increase

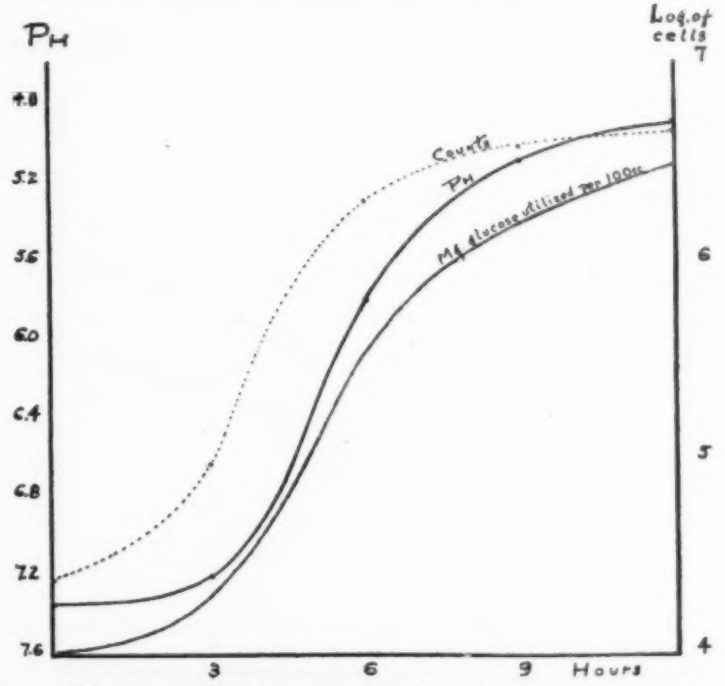

FIG. 3. EXPERIMENT III. CULTURE (2); 1 PER CENT GLUCOSE, 5 PER CENT HORSE SERUM BROTH (PLEURAL FLUID STRAIN)

in the output of ammonia a coincident decrease in amino acid production is evident. In the serum cultures the curves of amino acid output rise sharply at the sixth hour while in the glucose culture the rise is delayed until the ninth hour. The initiation of this rise appears to be in direct correlation with a high point of the ammonia curve.

From the results of this experiment it seems evident that acid formation is closely associated chronologically with growth and active metabolism of the streptococcus. In each of the three cultures we find the maximum period of acid production correlated with maximum rates of growth, and of glucose utilization. These results are not in accordance with the findings on pneu-

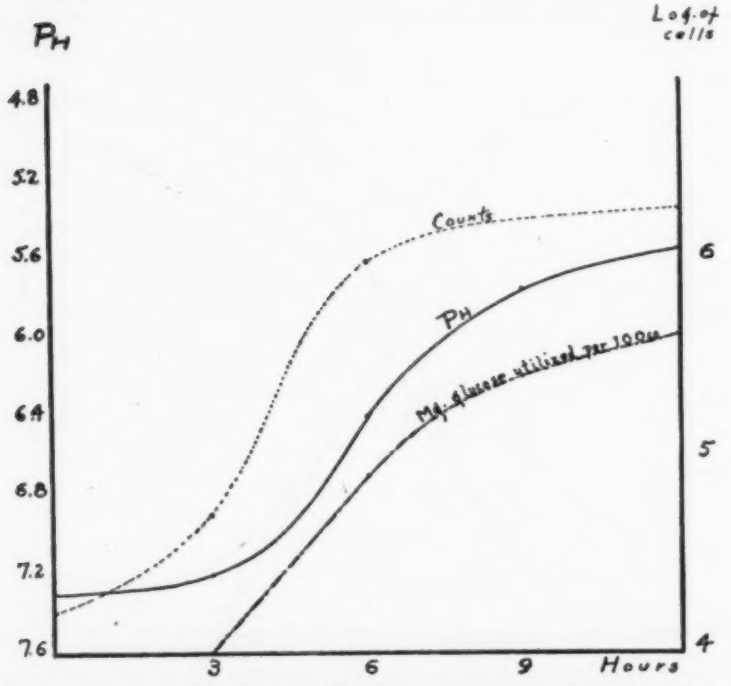

FIG. 4. EXPERIMENT III. CULTURE (3); 1 PER CENT GLUCOSE, 5 PER CENT HORSE SERUM BROTH (LABORATORY STRAIN)

mococci by H. M. Jones (1920) who reported a maximum period of growth correlated with slow acid formation, whereas the maximum period of acid production occurred during the time when the organisms were multiplying at a diminishing rate.

In a study of the nitrogen metabolism of actinomycetes, Waksman (1920) concluded that the production of amino acids

is not a waste process resulting from growth but that it represents a definite step in the metabolism of the organisms. In his experiments amino acids did not accumulate in the medium until after the organism had made its growth. In explanation Waksman suggests two possibilities; either (1) the growing cells utilized the amino acids as rapidly as the latter were formed,

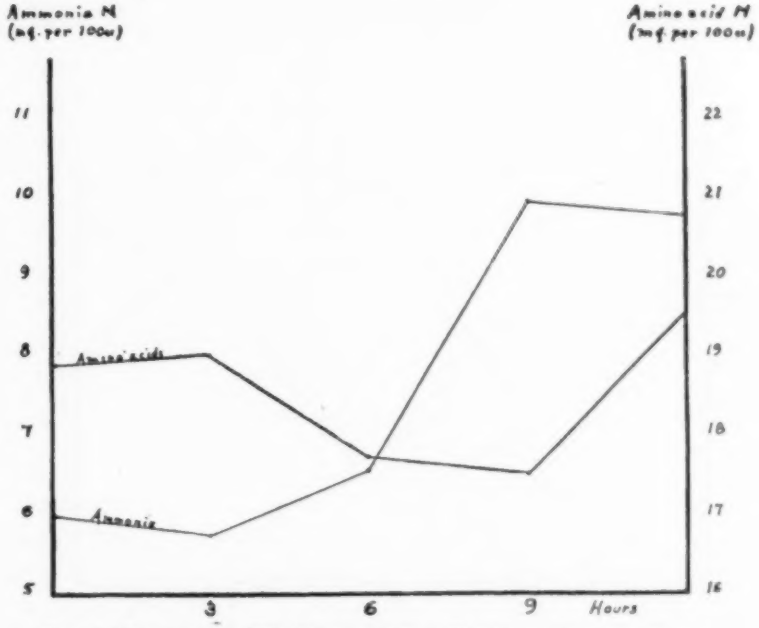

FIG. 5. EXPERIMENT III; CULTURE (1); 1 PER CENT GLUCOSE BROTH

or (2) the proteolytic enzyme necessary for their elaboration appeared only in the later stages of growth. Attention has been called to the fact that the curves of amino acid formation in experiment III exhibit a rise at the sixth or the ninth hour which would correspond to the findings of Waksman on the actinomycetes. Examination of the growth curves (figs. 2, 3, 4) at this point shows that the maximum period has just been passed and that the organisms are now multiplying at a diminishing

rate. As a consequence the amino acid intake of the cells is reduced to a low level. This would account for the increased output in the medium. It has been pointed out that the rise in ammonia production in each culture, starting at the third hour, is correlated with a drop in the amino acid curve (figs. 5, 6, 7). The most probable explanation of this finding rests

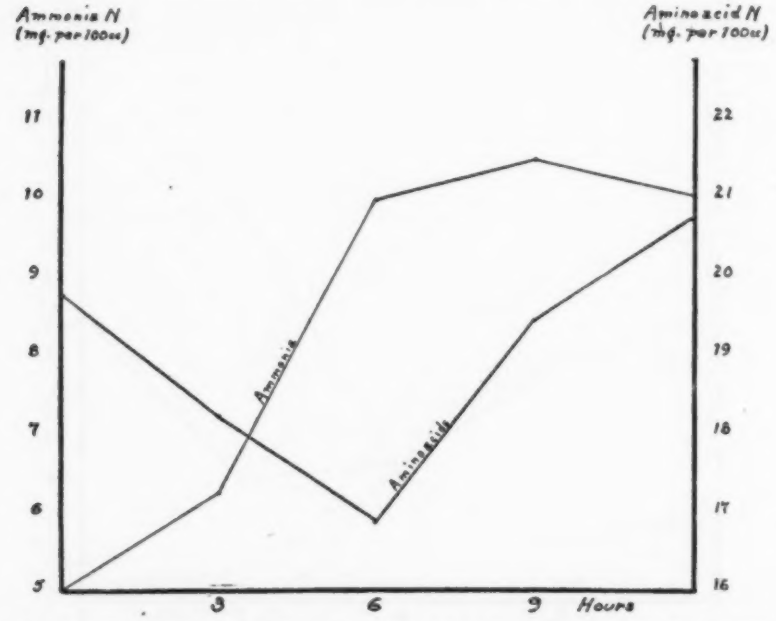

FIG. 6. EXPERIMENT III. CULTURE (2); 1 PER CENT GLUCOSE, 5 PER CENT HORSE SERUM BROTH (PLEURAL FLUID STRAIN)

upon the supposition that during the early life of the culture amino acids are utilized by the cells for structural purposes thus reducing their concentration in the medium. As more amino acid nitrogen is assimilated, a larger amount of ammonia is split off intracellularly. This is evidenced by a rise in the curves of ammonia production during this period. Such an hypothesis is in accord with the theory of Kendall and Walker (1915) that

ammonia formation is the result of intracellular deaminization of assimilated protein material.

Wolf and Harris (1917b) in their study of the biochemistry of *Clostridium welchii* and *C. sporogenes* noted in cultures grown in a medium of high amino acid content that at the close of the experiment the concentration of amino acids was less than at the begin-

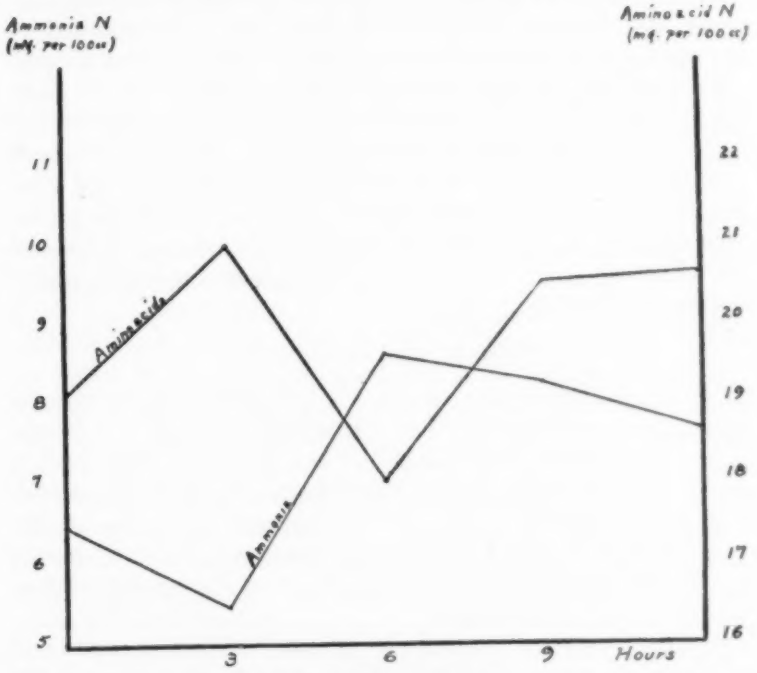

FIG. 7. EXPERIMENT III. CULTURE (3); 1 PER CENT GLUCOSE, 5 PER CENT HORSE SERUM BROTH (LABORATORY STRAIN)

ning. This indicates that these substances were assimilated by the growing organisms and destroyed through deaminization.

Reference to figures 6 and 7 reveals a very decided difference in nitrogen metabolism in the two serum cultures during the initial three-hour period. The pleural fluid culture exhibited a definite decrease in amino acid output correlated with an increased

ammonia formation, whereas the laboratory culture showed a decided increase in amino acid output coupled with a slight decrease in ammonia excretion. Whether or not this deviation represents a permanently altered aspect of metabolism on the part of the laboratory strain must for the present remain undecided. It may be that through continued cultivation upon artificial media the organism has gained the ability to inaugurate proteolysis earlier. Such a conclusion would be opposed to the finding of Rosenthal and Patai (1914) that avirulent streptococci were less strongly proteolytic than cultures of the same strain the virulence of which had been increased by animal passage. In the present experiment the curves of nitrogen metabolism (figs. 6 and 7) show the same general features from the third hour to the end of the period of observation. No differences in the growth, acid formation, or glucose utilization were evident in the two cultures throughout the entire period of the experiment.

SUMMARY AND CONCLUSIONS

1. Lactic acid appears to be the principal acid formed by *Streptococcus hemolyticus* in its fermentation of glucose broth. A smaller proportion of volatile acids is formed. This fraction is made up chiefly of acetic, with perhaps a trace of formic acid.

2. A quantitative study of the ammonia excretion of the streptococcus indicates that a "protein sparing" action occurs in media containing available sugar to meet the energy requirement of the developing cells.

3. The maximum periods of glucose utilization and acid formation in glucose and in glucose-serum broth are correlated with growth at a maximum rate, though a considerable lowering of P_H occurs during the period when growth proceeds at a diminishing rate.

4. The greatest increase in output of ammonia is correlated in a general way with the maximum periods of growth, glucose utilization, and acid formation. Associated with this increased output of ammonia a corresponding decrease in amino acids is evident. This condition seems to be associated with the interval

in which the organisms are making their growth. During this period, in which it may be presumed that anabolic processes are actively under way, the organisms are utilizing amino acids for structural purposes. This would cause the latter to decrease in concentration in the medium and furthermore would produce an increased excretion of ammonia through the katabolism of a part of the absorbed amino acids.

5. Subsequent to the period in which the organisms have made their growth a rise in the curves of amino acid formation is manifest. In the cultures containing horse serum this rise is initiated by the sixth hour; in the glucose culture it appears by the ninth hour. A decrease in ammonia output, in general, accompanies the rise in amino acid formation. These findings indicate that a decreased utilization of nitrogenous materials ensues after the organism has passed its maximum period of growth, despite the fact that proteolysis continues.

6. A marked difference in nitrogen metabolism between a passage strain and a laboratory strain of *Streptococcus hemolyticus* is noted during the first three hours of incubation in glucose serum broth. Whereas the passage strain shows a definite decrease in amino acid output coupled with an increased ammonia excretion, the laboratory strain exhibits a decided increase in amino acid output coincident with a slight decrease in ammonia formation. Whether or not this represents a permanent deviation in metabolism resulting from continued transplantation upon artificial culture media is a question that for the present must remain undecided.

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NOTES ON THE FLAGELLATION OF THE NODULE BACTERIA OF LEGUMINOSAE

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For many years it has been known that the nodule bacteria of Leguminosae are motile. Some early reports give the number of flagella as one, other reports describe several. From time to time some addition has been made to our knowledge of the flagellation of these organisms. Since the information on this point has been rather fragmentary and somewhat uncertain, the writer began the study of the flagellation of the nodule bacteria from a considerable number of host plants in the spring of 1920.

Different workers, using bacteria from different host plants, have found in some cases a single flagellum and in other cases several peritrichic flagella, and in a number of instances it has been assumed because the ones under observation were of a certain type, that therefore all nodule bacteria were similar to the ones studied. This point together with the general paucity of information on the flagellation of legume bacteria is well brought out by the following brief historical review of all available published reports.

Smith (1900) reported a single terminal flagellum about two μ long bearing at the end a tuft like the lash of a whip. He does not state the source of his organism.

DeRossi (1907) found a single flagellum on organisms from *Vicia faba*. He later (1920) reported the organism to be a true Bacillus with several flagella, but it is not clear from his account what host plants furnished the bacteria except that *Trifolium repens* was one of those used.

Harrison and Barlow (1907) reported a single polar flagellum on the organisms from *Lathyrus sativus* and *Vicia villosa* but their method of staining was such that only the slime and mucilage around the bacteria were stained, leaving the bacteria themselves unstained.

Zipfel (1912) reported numerous peritrichic flagella, but did not state the source of his organisms.

Kellerman (1912) reported numerous peritrichic flagella on the organisms from *Phaseolus lunatus*, *Medicago sativa* and *Pisum sativum*.

Burrill and Hansen (1917) have given us the most extensive information on the question of the flagellation of this group. They reported a single flagellum on organisms from *Vigna sinensis*, *Glycine hispida*, *Acacia floribunda*, *Arachis hypogoea*, *Baptisia tinctoria*, *Genista tinctoria*, *Cassia chaemacrista*, *Amphicarpa monoica*, *Lespedeza striata*, *Desmodium canescens*, and *Mucuna utilis*.

Fred (1918) has found on the alfalfa organism several peritrichic flagella and on the lupine organism one or rarely two.

Prucha (1915) has found several flagella on the organism from the Canada field pea.

Wilson (1917) reported as many as four flagella on the soy bean organism.

Hansen (1919) has found peritrichic flagella on the organisms from *Trifolium pratense*, *Vicia villosa*, and *Melilotus alba*.

Much of the difficulty in staining the flagella of these bacteria has been and still is due to the amount of slime that is produced by these organisms. Some produce slime more abundantly than others, and in some cases the mass of bacteria is so viscid that it may be drawn out in a string in making transfers.

METHOD OF ISOLATION

The roots of various leguminous plants were dug and carefully washed in running tap water. Small pieces of roots bearing nodules were then removed leaving enough root attached to aid in handling. These nodules were allowed to stand in tap water

for a few minutes after washing. With a pair of forceps a nodule was then placed in a disinfecting solution prepared by adding 2.5 cc. of concentrated hydrochloric acid to 500 cc. of a 1:500 corrosive sublimate solution and allowed to remain in this solution for one and a half to two minutes. It was then removed with a pair of flamed forceps, rinsed in sterile tap water, and placed in a drop or two of sterile tap water in the center of a sterile Petri dish. The nodule was crushed by using a flamed and cooled glass rod, after which a tube of sucrose agar which had been melted and properly cooled was added, and thoroughly mixed. The sucrose medium just referred to was made as follows:

Monobasic potassium phosphate	1.0 gram
Magnesium sulphate	0.5 gram
Sucrose	10.0 grams
Tapwater	1000.0 cc.
Agar	10.0 or 15.0 grams

At first no attempt was made to adjust reaction, but as the growth on this medium was so slow most of the media used were adjusted to pH 7.0–7.4 using the colorimetric method.

Several plates were made at each time, thus insuring good distribution of colonies in at least one of the plates. All plates were kept at room temperatures. After the colonies developed transfers were made either to the same sucrose medium or to a similar medium, containing 10 grams of mannitol in place of the sucrose. The mannitol media were used almost exclusively for maintaining the organisms after transfer from the isolation plates.

METHOD OF STAINING FLAGELLA

The staining method used was a modification of Loeffler's flagella stain suggested by the writer in a previous paper (1920). Bacteria from a slant on mannitol or sucrose agar were removed and placed in a small quantity of sterile tap water in a test tube. Several small droplets of this suspension were, after a few minutes, placed on a well cleaned cover glass and allowed to air dry. About five drops of Mordant solution A were placed on the cover glass as soon as the droplets had dried, and this was followed

immediately by one or two drops of solution B. The combination was allowed to act at room temperature for two minutes, washed in distilled water and the stain applied for two minutes. The excess stain was washed off with water and the cover glass preparation dried and mounted in balsam.

RESULTS OF STAINING

The age of cultures of the organism from different legume host plants made a considerable difference in the staining reaction. It was sometimes necessary on this account to try cultures of varying ages before successfully staining the flagella. The following table contains the data bearing on the source of the cultures, their age at time of staining, and the number of flagella.

DISCUSSION

Manifestly the flagellation of the legume nodule bacteria is of two types, the single flagellate type and the peritrichic. In every case in which the organisms possessed more than one flagellum the arragement was peritrichic and in the case of those with a single flagellum it was usually attached at the corner rather than exactly at the end. This corner arrangement seems to characterize the single flagellate type. In the case of organisms of this type, more than one flagellum was never found, and enough organisms were observed in each case so that there is no doubt that one is the correct number, and that it is an entirely different organism from the peritrichic type. Due to the breaking off of flagella in handling, bacteria of the peritrichic type showed an occasional organism with only one flagellum. For the most part the peritrichic flagella were longer than the single flagella and there was a tendency for the single flagella to be of greater diameter than those of the other type.

The present findings are in accord with those of Hansen (1919) who has suggested that since the organisms from different legumes have in some cases one flagellum, and in others several flagella, we have really two groups of organisms based on these differences of flagellation. Conn (1920) is of the opinion that the

TABLE 1

Flagellation of legume nodule bacteria

HOST PLANT	AGE OF CULTURE	NUMBER OF FLAGELLA
	days	
Vicia angustifolia (smaller common vetch)	2	3 to 4
Vicia dasycarpa (vetch)	4	3 to 4
Vicia hirsuta (tineweed or vetch)	4	2 to 4
Vicia alba (vetch)	1	3 to 5
Vicia sativa (common vetch)	3	1 to 6
Vicia villosa (hairy vetch)	4	1 to 4
Vicia caroliniana (Carolina vetch)	4	2 to 3
Trifolium pratense (red clover)	3	3 to 5
Trifolium procumbens (low hop clover)	5	2 to 6
Trifolium incarnatum (crimson clover)	2	2 to 6
Trifolium repens (white clover)	3	2 to 5
Trifolium dubium (least hop clover)	2	1 to 5
Trifolium arvense (rabbit-foot clover)	4	1 to 4
Trifolium hybridum (alsike clover)	2	2 to 4
Medicago arabica (bur-clover)	2	7 to 14
Medicago sativa (alfalfa)	4	1 to 4
Melilotus alba (white sweet clover)	3	5 to 8
Robinia pseudo acacia (locust tree)	3	2 to 4
Albizzia julibrissin (silk tree)	7	1
Cassia nictitans (sensitive pea)	5, 6	1
Falcata comosa (hog peanut)	5	1
Baptisia tinctoria (wild indigo)	5	1
Cracca virginiana (wild sweet pea)	2	1
Cracca spicata (loose flowered goat's rue)	4	1
Pisum sativum (garden pea)	2	4 to 9
Phaseolus vulgaris (garden bean)	2	2 to 6
Phaseolus lunatus (lima bean)	2	1 to 4
Soja max (soy bean)	5, 6, 10	1
Meibomia laevigata (smooth tick trefoil)	4	1
Meibomia viridiflora (velvet-leaved tick trefoil)	4	1
Meibomia obtusa (hairy tick trefoil)	4	1
Meibomia paniculata (panicled tick trefoil)	6	1
Vigna sinensis (cow pea)	14	1
Arachis hypogoea (peanut)	6	1
Stylosanthes biflora (pencil flower)	5	1
Clitoria mariana (butterfly pea)	4	1
Pueraria thunbergiana (kudzu vine)	5	1
Dolicholus erectus (erect rhynchosia)	2	1
Lathyrus odoratus (sweet pea)	5	2 to 4
Lespedeza striata (Japan clover)	6	1
Stizolobium deeringianum (velvet bean)	5	1

different results obtained by Wilson (1917) who found peritrichic flagella on the soy bean organism, and Hansen (1919) who found the single flagella on organisms from the same host, are due to the age of the cultures at time of staining. From inquiry Conn learned that Wilson's cultures were sometimes as old as twenty-eight days, whereas Hansen used two to three day old cultures. Conn suggests therefore, that the organism may be of the single flagellate type when two or three days old and becomes peritrichic when older. However, Wilson's paper (1917) states that the flagella were stained from one to seven day old cultures so the peritrichic flagella must have been present in seven days or less, and he furthermore makes no reference to finding single flagella in the younger cultures.

While the writer has been unable to stain flagella on the soy bean organism from very old cultures, yet he has found that up to ten days old the cultures still show the single flagellate type, with no indication that they will ever be anything else.

Wilson demonstrated that his peritrichic organism was able to form nodules on soy beans, and the strain of the single flagellate soy bean organism used by the writer has also been shown to be able to produce nodules when grown according to the method of Garman and Didlake (1914). This brings the writer to the conclusion that in different sections of the country, there is a different adaptation of nodule bacteria to the soy bean, and that Wilson and Hansen were working with the two different types.

Although a similar adaptation might be expected in the case of other legume host plants, yet it is interesting to note by referring to the groups of nodule bacteria found by Burrill and Hansen (1917) that if one host plant in a group has single flagella, all other host plants of that group which were investigated gave single flagella, and similar results were obtained in those groups having peritrichic flagella.

SUMMARY

1. The flagellation of the organisms from nodules of 41 species of leguminous plants has been studied.

2. Two distinct types of flagellation have been found, the single flagellate type and the peritrichic.

3. As suggested by Hansen (1919) the writer believes that the nodule bacteria of the Leguminosae are of two groups and if we follow Migula's classification they belong to two genera, Pseudomonas and Bacillus.

4. From 15 genera the flagella were of the single flagellate type. From 8 genera the flagella were of the peritrichic type.

5. In no case has any difference been found in the type of flagellation on organisms from plants of different species of a genus.

6. The single flagellate type is not strictly polar as the flagellum is usually attached at the corner rather than exactly at the end.

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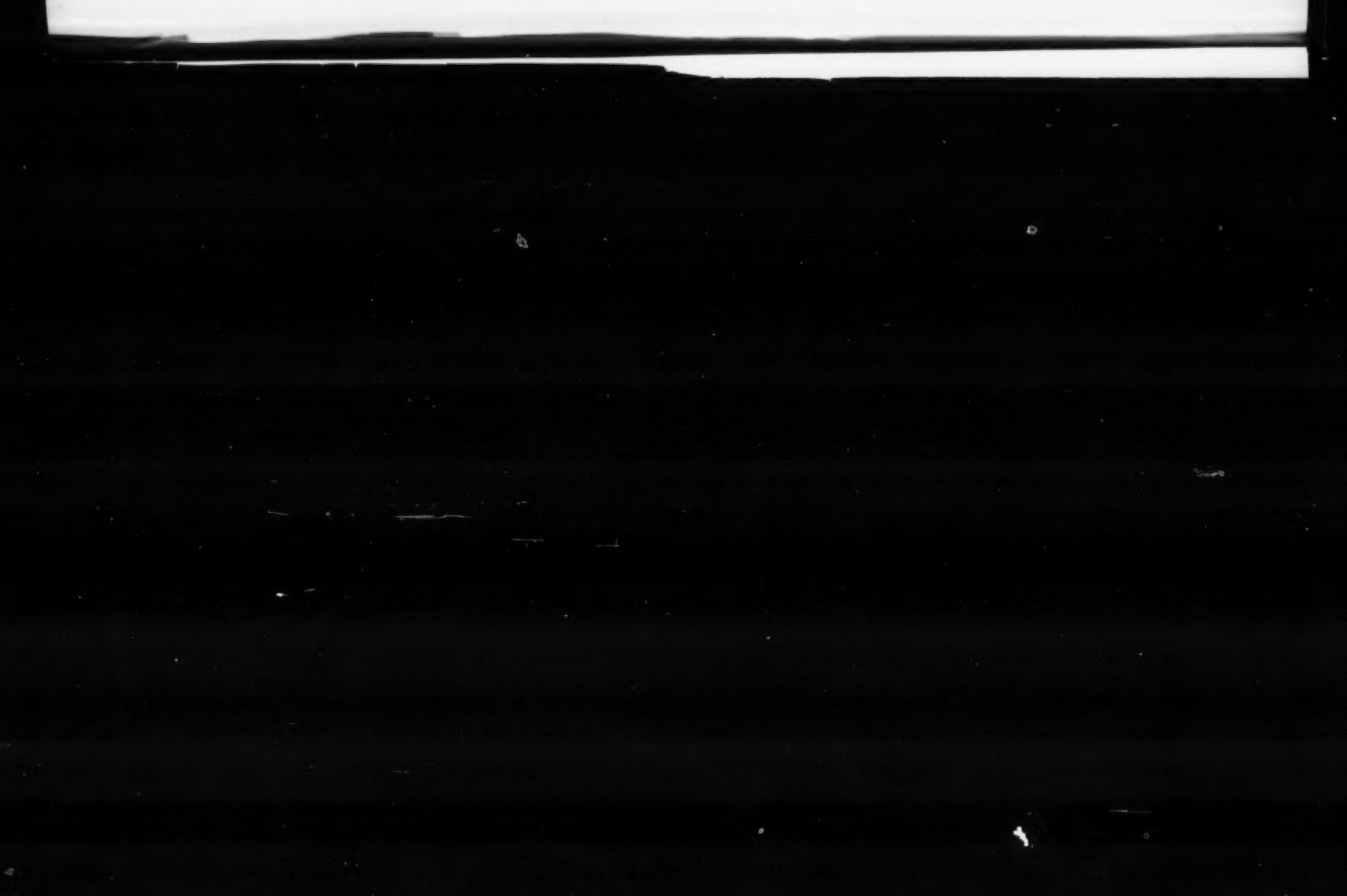
PLATE 1

FIG. 1. *Phaseolus vulgaris* (common bean)
FIG. 2. *Cracca virginiana* (wild sweet pea)
FIG. 3. *Vicia villosa* (hairy vetch)
FIG. 4. *Vicia caroliniana* (Carolina vetch)
FIG. 5. *Caissia nictitans* (sensitive pea)
FIG. 6. *Trifolium hybridum* (alsike clover)
FIG. 7. *Meibomia viridiflora* (velvet-leaved Tick Trefoil)
FIG. 8. *Trifolium dubium* (least hop clover)
FIG. 9. *Albizzia julibrissin* (silk tree)
FIG. 10. *Robinia pseudo acacia* (locust tree)
FIG. 11. *Stylosanthes biflora* (pencil flower)
FIG. 12. *Medicago sativa* (alfalfa)
FIG. 13. *Vicia sativa* (common vetch)
FIG. 14. *Pisum sativum* (garden pea)
FIG. 15. *Trifolium repens* (white clover)
FIG. 16. *Melilotus alba* (white sweet clover)
FIG. 17. *Trifolium arvense* (rabbit-foot clover)
FIG. 18. *Baptisia tinctoria* (wild indigo)
FIG. 19. *Vicia alba* (vetch)
FIG. 20. *Cracca spicata* (loose flowered Goat's Rue)
FIG. 21. *Vicia hirsuta* (tineweed or vetch)
FIG. 22. *Arachis hypogoea* (peanut)
FIG. 23. *Medicago arabica* (bur-clover)
FIG. 24. *Pueraria thunbergiana* (kudzu vine)
FIG. 25. *Vicia dasycarpa* (vetch)
FIG. 26. *Falcata comosa* (hog peanut)
FIG. 27. *Trifolium procumbens* (low hop clover)
FIG. 28. *Trifolium incarnatum* (crimson clover)
FIG. 29. *Lathyrus odoratus* (sweet pea)
FIG. 30. *Clitoria mariana* (butterfly pea)
FIG. 31. *Vicia angustifolia* (smaller common vetch)
FIG. 32. *Trifolium pratense* (red clover)
FIG. 33. *Meibomia laevigata* (smooth tick trefoil)
FIG. 34. *Meibomia paniculata* (panicled tick trefoil)
FIG. 35. *Dolicholus erectus* (erect rhynchosia)
FIG. 36. *Phaseolus lunatus* (lima bean)
FIG. 37. *Soja max* (soy bean)
FIG. 38. *Meibomia obtusa* (hairy tick trefoil)
FIG. 39. *Vigna sinensis* (cow pea)
FIG. 40. *Stizolobium deeringianum* (velvet bean)
FIG. 41. *Lespedeza striata* (Japan clover)

All drawings were made to the same scale and with the aid of a camera lucida.

(Shunk: Flagellation of Bacteria of Leguminosae)



METHOD FOR THE INTRAVENOUS INJECTION OF GUINEA-PIGS

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Up to the present time two well-known methods have been employed for the intravenous injection of substances into guinea-pigs, namely the jugular vein and the ear vein methods.

By certain investigators the first method is considered objectionable by reason of the fact that when the jugular vein is used it is exceedingly difficult to control the head of the animal without interfering with the operator's movements when making the injections. The marginal vein of the ear which is advocated by Rous[1] can be employed only in selected animals and therefore is not especially adapted for routine use.

A method for routine work, which seems to have a distinct advantage over the preceding methods has been elaborated recently. This makes use of the comparatively large superficial vein lying on the dorsal and inner aspect of the hind leg of the animal. This vein nearly always runs diagonally[2] across the leg from the dorsal aspect below to the inner aspect above.

To use the above vessel for intravenous administration an operating board (fig. 1) has been devised which permits the operator to manipulate the hind legs of the animal freely and at the same time does not prevent the legs from being securely tied. The board proper is made from a flat piece of wood and is 16 inches long, 8 inches wide and $\frac{3}{4}$ inch in thickness. It is similar to an ordinary animal board except that the end to which

[1] Rous, Peyton. Method for Intravenous Injection of Guinea-Pigs. Jour. Exper. Med., 1918, **27**, 459.

[2] Occasionally, the vessel may run anteriorly.

the hind legs are tied has a U-shaped piece cut from it, as shown in the illustration. The board is mounted near its center on an extension shaft, which is fitted with two joints, the one at the end to which the board is attached being a ball and socket joint and the other an adjustable swivel joint. The shaft is screwed into a metal base which has sufficient weight to hold the board steadily when placed in any position.

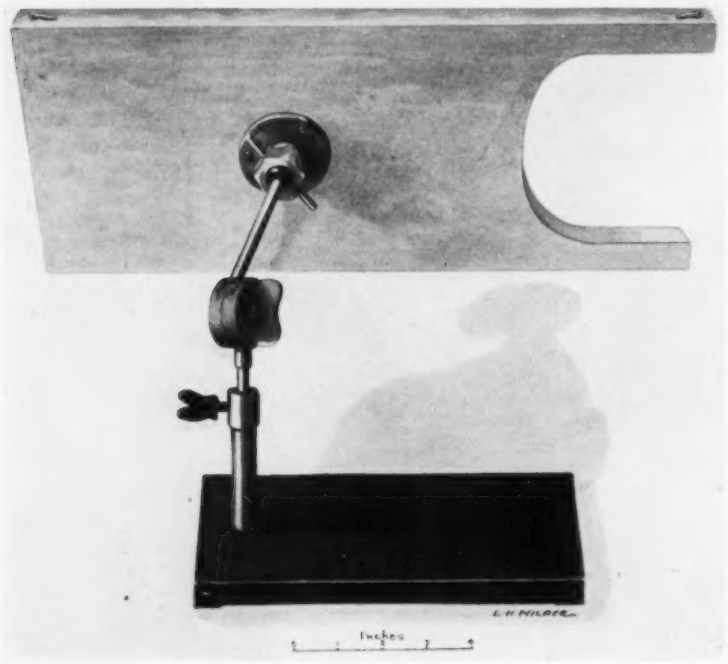

FIG. 1

The procedure for making the injection is as follows: With the board proper placed in a horizontal position, the animal is tied to it securely, abdomen downward, by means of strings. While the animal is being anesthetized the board is placed in a vertical position and rotated on its vertical axis slightly so as to bring

the dorsal aspect of the right hind leg into view (fig. 2). After clipping the hair from the leg and shaving it, the leg is lifted up slightly by the first, or first and second fingers and lightly compressed by the thumb of the left hand. A small incision, usually about $\frac{1}{4}$ inch long is made diagonally across the leg from the

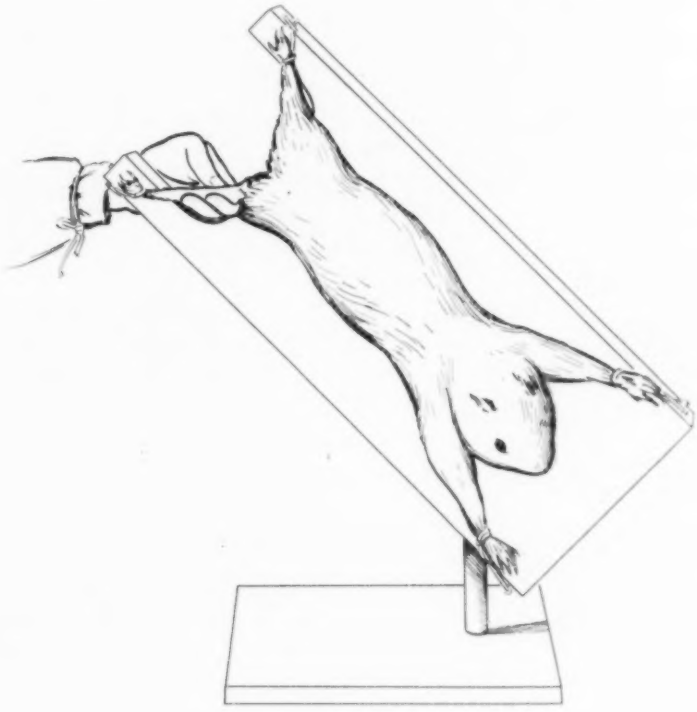

FIG. 2

outer, lower to the upper and inner aspect (fig. 3). The subcutaneous tissue is then pushed aside with a fine pointed forceps thereby permitting the vessel to come into view. The vessel when dilated by suitable compression permits the ready entrance of a number 23 B. & S. gauge needle, although a number 26 B. & S., $\frac{5}{8}$ inch needle is usually employed. The needle is always

introduced well into the lumen of the vein so that if there is any escape of fluid backward it can be prevented by pressure on the

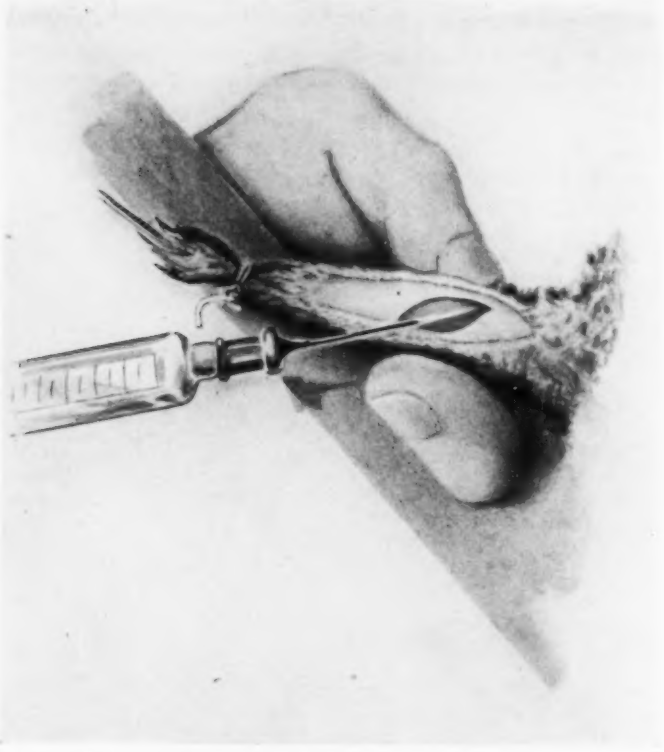

FIG. 3

vessel between the point of the needle and the opening in the vessel. Bleeding from the cut vessel can be readily controlled by pinching it with small forceps.

ROSE BENGAL AS A GENERAL BACTERIAL STAIN

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Recently the use of rose bengal was mentioned (Conn, 1918) as a stain for detecting microörganisms in the soil. It has subsequently been found to be especially useful in staining two soil organisms, *B. radicicola* and *Ps. caudatus*, both of which are hard to stain by ordinary methods on account of the slime they produce. Its use with these two species proved so successful that it is now one of the bacterial stains most commonly used in this laboratory, and it almost always gives satisfaction.

Rose bengal is a stain little known among bacteriologists, but deserves wider use. It belongs to the phthalein series of dyes, being closely related chemically to eosin. It differs from the latter stain in that the four atoms of bromine in eosine are replaced by four atoms of iodine with the addition of two atoms of chlorine. The dye as formerly made in Germany was apparently quite reliable, but it has scarcely ever been manufactured in America and it has proved difficult to get a satisfactory product of domestic manufacture. Some of the imported material is still available, and after investigation an American-made product has been found, sold by the Will Corporation as their "bioloid" grade, which gives as good results as that of German manufacture. The formula which has proved satisfactory is: Rose bengal, 1 gram; 5 per cent phenol, 100 cc. This solution remains in good condition for several months.

The chief value of this stain to the bacteriologist is that it has a great affinity for bacterial protoplasm but not for the slime or débris with which the cells are surrounded. It is therefore especially to be recommended for slime-formers. *B. radicicola*, for instance, does not take the ordinary bacterial stains

unless it has been carefully separated from its slime, the organisms remaining unstained in a completely stained field. With rose bengal, on the other hand, the organisms stain deeply, showing their characteristic granules, and the field is practically unstained even though the organisms have been growing on a medium specially adapted to the production of slime. *Ps. caudatus*, which is only about 0.2 micron in diameter (see Conn and Bright, 1919), shows a peculiar and typical granulation with this stain that had not been suspected after considerable study with other dyes. Rose bengal, indeed, seems to be unusually well adapted for revealing structure in small bacterial cells. The very satisfactory results obtained with it in this laboratory make it seem worth recommending to bacteriologists in general. Although more expensive than the more commonly used dyes, its price is not prohibitive unless an unusual amount of staining is to be done.

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